THE STRUCTURE AND PROPERTIES OF SYNTHETIC POLYPEPTIDES AND RELATED BIOMOLECULES

by

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SUMMARY

The structure and properties of synthetic polypeptides and related biomolecules have been investigated in the solid state, in solution and at interfaces, by a diversity of physical methods.

Studies of polymers in the solid state and in solution were the first to show that the α-helix normally exists in only one screw sense and that it is right handed. Work on poly-glycine has led to advances in our knowledge of its structure and that of related proteins.

A range of polymers spread as molecular monolayers at the air-water interface has been investigated by combining the classical methods of surface chemistry with peptide deuterium exchange used as a structural tool, and by examining collapsed films by electron diffraction and polarized infrared spectroscopy. Contrary to the conclusions of others working in this field, it has been shown that many high molecular weight polypeptides appear to be stable at the air-water interface in the α-helical conformation. A transition observed in the monolayer state has been shown to be consistent with the regular collapse of the structure to form a bilayer, rather than a conformational change; this transition gives valuable quantitative information relating to the molecular forces involved. There is good evidence that the surface potential arises from polymer-water interactions rather than, as has been supposed, from the orientation of the peptide dipoles with respect to the interface. Certain

polymers have been found which have unusual properties. In one case a polymer which normally forms a left handed helix appears to form a right handed helix in the monolayer state; in another case a series of transitions has been observed consistent with the consecutive formation of several layers of molecules. This work provides additional confirmatory evidence for the interpretation of the monolayer properties. Polymer-water interactions studied by polarized infrared radiation have shown that water molecules adsorbed from the vapour are located in specific orientations; this has opened up a valuable method for studying water-peptide group and water-side chain interactions, and supports the molecular basis of the interpretation of the surface potential.

The properties of proteins and synthetic polymers have been examined in relation to the conformation of proteins at interfaces and in cell membranes. This work was the first to show that infrared spectroscopy and optical rotatory dispersion could be applied to test the idea generally accepted, that the proteins were in the extended β-conformation. It has been shown that this view is without sound foundation and that the application of these methods can provide new information of considerable value.

PREFACE

My interest in the synthetic polypeptides developed from my appointment as a research physicist to the Fundamental Research Laboratories of Courtaulds Limited at Maidenhead, England, in October 1951, where pioneer work on these polymers had already led to the laboratory being internationally recognised. In particular it had been shown that there was a close parallel between the α and β forms of natural proteins, as designated by Astbury, and similar conformations in the synthetic polymers. The laboratory was altogether exceptional for an industrial organization in its liberal approach to science. While it was always central to our work that a synthetic fibre might emerge having all the desirable textile properties of the natural protein fibres, wool and silk, a fundamental interest in the properties of both natural and synthetic polymers and publication of research was actively encouraged; my first paper from the laboratory was in fact on the infrared spectrum of frog sartorius muscle.

Of the individuals working within this field at the laboratory to whom I am particularly indebted mention must be made of three: C.H. Bamford who was in charge, and mainly but far from exclusively concerned with the physical chemistry; W.E. Hanby a skilled organic chemist whose ability in producing polymers with the properties we desired was crucial; finally A. Elliott, with whom I worked most closely, a dedicated researcher, whose spectroscopic skills ranging

from the infrared to the X-ray region of the spectrum are internationally recognised. The total number of scientific staff working on all aspects of synthetic polypeptides at the laboratory was seldom more than six or eight, nevertheless we made rapid progress, not only because the field was fruitful, but also because we came from a diversity of scientific backgrounds which was central to our success. We formed a group which would be quite exceptional in a university department.

On my appointment as a Lecturer in the new Department of Biophysics of the University of Edinburgh in January 1958, until that time a Unit of the Department of Natural Philosophy, I expected to move away from work on synthetic polypeptides. The main line of research in the laboratory, associated with J. Dainty, was ion transport in cells. To develop an independent line of research, but perhaps related to the other work of the laboratory, it seemed useful to work on the structure and properties of interfaces such as cell surfaces, known to be central to the osmotic and ion transport properties of cells. A direct approach to this problem was, however, restricted by the inadequacy of the experimental techniques available, and corresponding ambiguities in the interpretation of the data. It therefore seemed perhaps more profitable to follow up the study of the properties of proteins at interfaces along the lines pioneered by N.K. Adam, J.F. Danielli, I. Langmuir and E.K. Rideal

among others. The techniques they had used and the ideas they developed had remained relatively static compared with the advances in our knowledge of proteins in the crystalline state. A reappraisal of the early surface chemistry of proteins was therefore timely, but there seemed no reasonable possibility that developments in monolayer methods utilising proteins, would immediately produce advances comparable to those made in the study of crystalline systems. I therefore returned to the study of synthetic polypeptides, this time in the monolayer state, with the expectation that as in the fibrous state, they would provide a bridge between the complexity of proteins and a system amenable to physical studies, on which new methods could be tried and tested.

In the notes on the papers that follow it has been my aim to try to fill in the background to the papers and comment on them in retrospect, to show how they were influenced by the problems (and personalities) of the time, and how they have contributed to the advancement of knowledge and experimental methods in their field. It is hoped that this will add interest to the papers and make them more readable, and also reflect the human element which is seldom allowed to appear in published work but which is an inextricable part of all research.

As far as possible the papers are considered in groups. Where the work was undertaken in collaboration with other experts, so far as I have been able the part I played and that of others is delineated in a paragraph at the end of the notes on each section, to conform with the Regulations of the University of Edinburgh. It will be appreciated that

to do this in detail is almost impossible in some instances, since the close interactions often essential in a collaborative piece of work are far too complex to unravel, and in these instances I would not wish to claim more than my fair share of any credit.

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CONTENTS

LIST OF PAPERS

1. "The near infrared absorption spectrum of some natural and synthetic fibres". With A. Elliott and W.E. Hanby, Brit.J.Applied Phys., 5, 377-381, (1954).
2. "Some Observations on the Infrared Spectrum of Muscle". Symposia of the Society for Experimental Biology, No. IX, 265-270, (1955).
3. "The mounting of fibres and organic crystals for spectroscopy in the near infrared region". J.Sci.Inst., 35, 423-424, (1958).
4. "Absolute Configuration and Optical Rotation of Folded (α) Synthetic Polypeptides". With A. Elliott, Nature, 178, 912, (1956).
5. "Chain arrangement and sense of the α-helix in poly-L-alanine fibres". With A. Elliott, Proc.Roy.Soc. A 249, 30-41, (1959).
6. "A sensitive photoelectric polarimeter". With A. Elliott, J.Sci.Inst., 34, 48-49, (1957).
7. "Optical Rotation of the α-Helix in Synthetic Polypeptides". With A. Elliott and W.E. Hanby, Nature, 178, 1170, (1956).
8. "Optical Rotation and molecular configuration of synthetic polypeptides in dilute solution". With A. Elliott, W.E. Hanby and A.R. Downie, Proc.Roy.Soc. A 242, 325-340, (1957).
9. "Structure and properties of synthetic polypeptides and silk proteins". With A. Elliott, Trans.Faraday.Soc., 52, 528-536, (1956).
10. "Structure of polyglycine". With C.H. Bamford, L. Brown, E.M. Cant, A. Elliott and W.E. Hanby, Nature, 176, 396, (1955).

11. "Configurations of silk fibroins and synthetic polypeptides". With A. Elliott, Biochim.Biophys.Acta., 21, 466-471, (1956).

12. "Optical rotation and infrared spectra of some polypeptide and protein films". With A. Elliott and W.E. Hanby, Faraday Society Discussion No.25 167-172, (1958).

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15. "Structural changes and molecular forces in a compressed synthetic polypeptide monolayer". Polymer, 7, 595-602, (1966).

16. "Molecular structure and deuterium exchange in monolayers of synthetic polypeptides". Proc. Roy. Soc. A 305, 363-385, (1968).

17. "Right-handed α-helix and conformational changes in poly (β-benzyl-L-aspartate)". Nature, 219, 929-930, (1968).

18. "The surface chemistry of poly (β-benzyl-L-aspartate)". Biopolymers, 9, 911-922, (1970).

19. "Multilayer formation by a compressed monolayer of poly(ε-benzyloxy-carbonyl-L-lysine)". Biochem.J. 110, 733-737, (1968).

20. "Protein conformations and interactions at interfaces". S.C.I. monograph No.19, 102-121, (1965).

21. "Protein conformations in the plasma membrane". With A.H. Maddy, Science, 150, 1616-1618, (1965).

22. "Protein conformations in biological membranes". With A.H. Maddy, Science, 153, 213, (1966).

23. "Infrared absorption spectrum of water adsorbed on α-helical synthetic polypeptides". Nature, 227, 1358, (1970).

24. "Studies of synthetic polypeptide-water interactions using monolayer techniques". A.C.S. Polymer Preprints, 11, 1327-1333, (1970) - as revised for final publication.

INTRODUCTION

The starting point for this work was the study of high molecular weight synthetic polypeptides as materials closely allied to natural proteins, in relation to their undoubted scientific and possible technological importance. Polymers can be prepared with a wide range of properties determined principally by the appropriate choice of side chain. Many are soluble in water or organic liquids, others are almost completely insoluble. With skill, some can be made into strong fibres closely resembling silk or wool. While technical and economic considerations may have prevented their large scale production for the synthetic fibre industry, many are now available as fine chemicals from the U.S.A. and Israel. This is almost entirely a result of their purely scientific interest in the fields of biochemistry, medicine and the physical chemistry of high polymers. Their importance in these fields will be seen by reference to the standard work on these polymers by Bamford, Elliott and Hanby (1956) and the books edited by Stahmann (1962) and Fasman (1967).

In my work I have had three main aims. Firstly to develop an understanding of the properties of synthetic polypeptides in the solid state, in solution and at interfaces. Secondly to attempt to relate where appropriate such results to proteins. Finally throughout this work it has been my interest as a physicist to develop and apply new methods to these studies, to extend our knowledge and bring to light new phenomena.

The value of these polymers as protein analogues arises from their relative simplicity which enables us to highlight features of proteins that would otherwise be difficult to isolate and study in detail. In some instances their properties are unusual and we find ourselves studying unexpected aspects of molecular behaviour. A number of factors are important: (i) the correct choice of polymers and their availability, (ii) studying them in the appropriate state and environment, and (iii) the use of all available methods in order to build up a detailed and self-consistent picture of their properties. We can then with caution extend the ideas and methods to proteins.

It was logical that in the first instance attention should be given primarily to the solid state and solutions, in order to develop our ideas about molecular structure, and this remains the central field of research on polypeptides, with relatively few attempts to study their properties in other situations. In particular since many are insoluble in water, this has been seen as a limitation to their usefulness in relation to proteins. This can to some extent be overcome, or even turned to advantage, by studying their properties as monolayers at the air-water interface. There is increasing realisation of the importance of the hydrophobic regions of proteins, and monolayer studies are of considerable relevance as a method of bringing hydrophobic side chains and water into close proximity. Clearly this approach is also of value in relation to

the surface chemistry of proteins and the study of interfacial structures generally. Yet another method is to study the adsorption of water on the polymers from the vapour phase. I have been particularly concerned to extend our knowledge into these fields.

How the results from these studies can or should be applied to proteins is a matter for scientific judgement. There is no doubt that in many respects they have clarified our thinking, for instance in relation to the secondary structure of proteins. Occasionally there has perhaps been a tendency to over-extend correlations between synthetic polymers and proteins, and sometimes a relation between the two has not been recognised when it should have been. My own attitude in this respect has developed towards studying the physical chemistry of the synthetic polymers as a worth while activity in its own right, but seeking to develop it along lines that might be expected to contribute to the better understanding of biological systems. To some extent it is a question of timing; at a particular state of development of this field one particular aspect of study of the synthetic polymers may produce a major advance in the study of proteins, for example in understanding optical rotatory dispersion, after which it becomes predominantly of physico-chemical interest. One might then be more profitably employed, from a biological standpoint, in some other aspect of the study of the synthetic polymers. The emphasis now, should be towards the problems presented by biological systems, as opposed to individual molecular species, with intermolecular

interactions, as well as reactions, and with aqueous systems. In general terms this is the direction in which my work has been aimed.

The work presented here started at a time when Elliott and Ambrose (my predecessor at the laboratory) had established the basic relationships between the conformations of the polymers and their polarized infrared spectra. It was then appropriate to extend this work to a broader range of polymers and to test the ideas that were emerging concerning molecular conformations.

SECTION I

The near infrared absorption spectrum of some natural and synthetic fibres, and of muscle (Papers 1, 2 & 3).

Paper 1. The need to prepare specimens sufficiently thin and uniform limits the diagnostic use of infrared spectroscopy in the 700-4000 cm^{-1} region, particularly in the examination of fibrous specimens. The near infrared 4000-6000 cm^{-1}, is then of value since specimens can be up to 1 mm in thickness, and sample preparation can be relatively simple (Paper 3). Most commercial instruments have a poor performance in this region and it is still relatively neglected. Its value is clearly shown by its use in enabling a comparison to be made of the molecular structure and orientation of very fine spider silk and thick fibres of poly-L-alanine. The regenerated protein fibres show almost no dichroism, strongly indicative of a low degree of molecular orientation, a fact doubtless connected to their subsequent commercial demise.

Paper 2. From a biological standpoint a further advantage of this region is that spectra can often be obtained in the presence of water. This enabled the spectrum of live muscle to be obtained. Pauling and Corey had suggested that the basis of muscle contraction was a change in the molecular conformation from the extended β-form to the

folded α-helical structure. A.V. Hill suggested that this could be tested in our laboratory and provided advice on the selection of a suitable muscle. The absence of a band at 4520 cm^{-1}, observed in the β-conformation of poly-L-alanine, (Paper 1) showed clearly that there were no grounds for the proposal. The spectrum also provided an indication that a small proportion of the protein was oriented in the α-helical conformation.

Paper 3. The method described for obtaining polarised infrared spectra of single crystals was developed in order to obtain spectra of various hydrated forms of cyclo-(hexaglycyl). This work remains unpublished, for while deductions could be made concerning the orientation of the peptide groups, there were ambiguities in the interpretation and it became evident that sooner or later a full structure determination by X-ray diffraction would emerge not subject to these uncertainties. Polarized infrared spectroscopy as a structural tool is of most value in systems not amenable to X-ray diffraction methods. However one feature of interest in relation to later work (Paper 23) was the observation of dichroism in the band associated with the water of crystallization.

(In Paper 1, I was principally responsible for the construction of the infrared spectrometer with the fused quartz prism, the mounting of the specimens, and with A. Elliott in interpretation of the results).

SECTION II (Papers 4 & 5)

The α-helix

Many accepted rather readily the α-helical structure proposed by Pauling, Corey and Branson as a basic structure for folded synthetic polypeptides and proteins. Their strong advocacy of this structure and its elegance were very persuasive. At a Royal Society Discussion (Astbury et al. 1953) it was clear that if it were correct, it raised a number of problems in the interpretation of the experimental data, to which the answers were by no means obvious. Poly-L-alanine was the ideal polymer on which to test the structure rigorously since it has a short side chain and could be prepared as fibres having a high degree of molecular orientation and crystallinity. A careful examination of the intensities of the X-ray diffraction pattern (Brown & Trotter , 1956) showed, however, that neither the left nor the right handed helix gave a satisfactory agreement with the data, though the left handed helix was on balance closer.

Elliott and I decided to re-examine the data using an optical diffractometer. It soon became evident that small modifications to the structure, compatible with reasonable bond lengths and angles and with the restriction of a 1.5 Å screw axis repeat, would not produce satisfactory agreement with the X-ray intensities; something more fundamental was involved. There was the possibility that both

enantiomorphs might be present (perhaps in different phases in the same specimen). However, discounting theoretical predictions that one sense of helix was significantly more stable than the other, we had found strong evidence for this from work on solutions of poly-leucine (Papers 7 & 8). It then became evident that a simple unit cell containing one molecule was not the correct choice, but that the structure probably consisted of a random arrangement of equal numbers of chains running in opposite directions, with the side chains packed in approximately equivalent positions. (A similar type of structure was subsequently found in poly (γ-methyl-L-glutamate), Vainshtein & Tatarinova, 1961). The random element in this structure also satisfactorily accounted for streaks on the layer lines, and it was then quite clear that the righthanded helix was present. It is interesting to reflect that what up to that time had been accepted as the diffraction pattern of an α-helix, was in fact the pattern that would be produced by a virtual structure consisting of two such helices running in opposite directions superposed, with the side chains in register. This virtual structure had a centre of symmetry, which simplified the phase problem, and we were able to propose slight modifications to the structure to improve the correlation with the X-ray data.

A recent re-examination of the X-ray photographs (Arnott & Wonacott 1966, Arnott & Dover 1967) has fully confirmed this work, even to the kind of detailed modifications that we suggested. However, from the use of a computer programme incorporating constraints relating to the bond lengths and angles, these modifications were accomplished without the need to deviate from the usual values.

(It is particularly difficult in retrospect to demarcate our respective contributions to this work. Appreciation of the random element in the structure came to us gradually as we worked on the problem. At a later stage I was mainly concerned with improvements to the original coordinates and in considering the packing of helices that did not have an exact repeat. Elliott was more concerned with interpretation of the layer line streaks).

SECTION III (Papers 6, 7 & 8)

The Conformation of Synthetic Polypeptides in Solution.

Paper 6. We were first led into studying the optical properties of solutions to obtain evidence for there being only one sense of helix. Visual optical polarimeters appeared to us to be rather archaic and beset with inconveniences which could be avoided with a photoelectric instrument and we therefore designed and constructed our own. It was simple and worked well, a second model was constructed for routine use by others in the laboratory and similar ones subsequently made commercially by Stanley Instruments Ltd. At a later stage we converted the quartz prism infrared spectrometer (Paper 1) for use as an ultraviolet monochromator and the combination enabled us to carry out optical rotatory dispersion measurements.

Papers 7 & 8. Poly-leucine was chosen for our first study since we felt reasonably certain it would be helical in non-polar solvents and because its side chain did not contain groups that might complicate its properties. The results it gave were pleasantly free from ambiguity and greatly clarified our ideas. Corresponding behaviour was found in a range of other polymers and it was clear that the sense of the helix was almost certainly the same in all

cases (Paper 8). Poly-alanine solutions were among these so that we considered it reasonably certain from the X-ray work that the helices were right handed. A final link between the two methods was made by examining the optical rotatory properties of some solid specimens of poly-alanine (Section IV, paper 12). This was of particular importance in connection with theories of optical rotation by Moffitt (1956) and Fitts and Kirkwood (1956) which predicted a right handed helix, though their basis for this was later seen to be insecure.

The value of our approach, combining X-ray diffraction with optical studies on solutions, and not relying on theoretical predictions concerning the optical properties, was not everywhere recognised, though Sheraga (1961) pointed out the merits of this work and Figure 1 (Paper 8) has been widely reproduced (e.g. Shooter, 1960, Schellman & Schellman, 1964, Morawetz, 1965). Doty (1957) referring to Blout, Doty and Yang (1957) was fully aware of Paper 7 when he wrote: "The first evidence for the existence of only one screw sense in the helix came from measurements of the optical rotation of copolymers of the L and D isomers of γ-benzyl-L-Glutamate". Similarly Yang (1967) remarks: "A decade ago the handedness of the helices was a rather controversial subject. Ironically the early premature prediction of the Moffitt theory for

a right handed α-helix happens to be correct for all the "standard" L-polypeptides. Confirmation comes from X-ray studies of myoglobin, hemoglobin and now lysozyme, all of which are right handed". And again: "Had we first studied helical poly(β-benzyl-L-aspartate), which has a positive b_o instead of poly(γ-benzyl-L-glutamate) in the early 1950s, a left handed helix would have erroneously been proposed for the L-polypeptides". (But see also paper 17!)

(In paper 6, the basic principle of the instrument was Elliott's suggestion. I was mainly responsible for the design and construction; we both took part in setting up and testing the instrument. In papers 7 and 8, Hanby prepared the polymers and Elliott and I made most of the measurements. Downie participated in the later stages of the work and was mainly responsible for the analysis relating to Figures 7 & 8 (Paper 8).)

SECTION IV (Papers 9, 10, 11 & 12)

The structure and properties of some synthetic polypeptides in the solid state and their relation to silks and other proteins.

Poly-L-alanine and poly-glycine and their copolymers were of interest not only because of their potential fibre forming properties, being closely related to silk, but also because their small side chains produced a simplification of the theoretical considerations in relation to infrared frequency analysis and X-ray work. These polymers did however present other problems, often of a practical nature. Poly-L-alanine tended to be very insoluble; poly-glycine was difficult to prepare with a sufficiently high molecular weight for specimens to be produced with a high degree of orientation, almost crucial for detailed X-ray analysis or polarized infrared studies.

Another odd feature of poly-glycine was that it never appeared to develop the α-helical structure, in contrast to poly (α-amino-iso-butyric acid)(in which the CH_2 group is replaced by $C(CH_3)_2$). It seemed likely that conditions most favourable to the formation of an internally hydrogen bonded helix would be obtained by precipitation of the polymer from very dilute solution. The conformation so produced was found to have an infrared spectrum

quite unlike those of other polymers (Paper 9), and the X-ray powder photograph corroborated the impression that the conformation was one not hitherto encountered. Because the only method of preparing this material was by precipitation, attempts to orientate it for studies by polarized infrared and X-ray diffraction were unsuccessful and it was therefore decided to publish a note drawing attention to its features (Paper 10). Crick and Rich almost immediately proposed the correct structure; one with intermolecular hydrogen bonds and a 3-fold screw axis (Crick and Rich, 1955). At that time a structure for poly L-proline with a 3-fold screw axis had been described by Cowan and McGavin (I believe at a conference in Yugoslavia attended by Crick), and published shortly after Paper 10, (Cowan & McGavin, 1955). Crick realised that if it were possible to show that poly glycine II also had a 3-fold screw axis, the structure of collagen was then almost self-evident. So it proved, the poly-glycine II providing the clue to the pattern of hydrogen bonding.

The structure proposed by Crick and Rich for poly-glycine II was not one that could accommodate side chains larger than hydrogen and it was therefore largely irrelevant to understanding the structures observed in silks, which depended not only on the source, which affected the amino acid composition, but also on how the material was treated. As with the near infrared region, we were able to relate the infrared bands of silk in its β-conformation

to poly-L-alanine and poly glycine in the same conformation, and identify bands arising from the side chains.

Problems arose in the interpretation of the spectra of silks prepared from solution in the absence of strain. While these had an Amide I band at about 1660 cm^{-1}, close to that of the α-helix, and in some cases there was corroborative X-ray evidence for the α-helical structure being present, in other cases neither X-ray nor optical rotatory dispersion studies gave any clear indication of a regular structure. I tended to regard these as glassy or random structures whereas Elliott considered it possible that the structure had some regularity. The conclusions of Papers 9 and 12 reflect our slightly different views. It is unfortunate that this work caused Astbury (1958) to state that we "once and for all abandon the exciting generalization to the effect that the α-helical configuration could be diagonised always by a carbonyl stretching frequency round about 1660-1665 cm^{-1} as opposed to 25-30 wave numbers fewer for the corresponding band given by the β-configuration. This criterion would have been invaluable, but from the beginning doubts arose till eventually, even before the coup de grâce was administered at this meeting came the unanswerable exception of poly glycine II for which they found 1648 cm^{-1}..."

Astbury failed to recognise that while specific conformations give rise to characteristic frequencies (sebsequently given a theoretical basis by Miyazawa, 1962) the frequencies are not necessarily unique

and may occur in other conformations; where a band occurs at a significantly different frequency, as with poly-glycine II, or is clearly not at the frequency associated with a proposed conformation, then it is useful evidence in recognising that some other type of structure is present. I make the same logical point at the end of Paper 2 in relation to the corresponding frequencies in the near infrared, where poly-glycine II has in fact a band coincident with α-helical structures.

We could however be criticised in retrospect for failing to recognise that the broadness of the Amide I band in lysozyme might include unresolved contributions from the presence of small amounts of both the α and β conformations, rather seeking to exclude them entirely (Paper 12).

(In this section Hanby prepared all the synthetic polymers, Elliott worked predominantly on the silks, whereas I was mainly concerned with the synthetic polymers, particularly poly-glycine. In paper 10 Brown and Cant were involved with the X-ray work, and Bamford mainly but not exclusively with attempts to increase the molecular weight of the polymer.)

SECTION V (Papers 13-19)

The structure and properties of synthetic polypeptides at the air-water interface.

Early work on proteins had clearly shown that at an air-water interface, proteins were frequently denatured and unfolded in some manner. Usually the molecule was depicted as a fully extended polypeptide chain (see for example Adam, 1941) with a β-keratin structure. When synthetic polypeptides became available their monolayer properties were interpreted in a similar manner. The review by Cheesman and Davies (1954) contains no suggestion that perhaps the α-helix might be present in some cases, despite interest in it at that time, and it was first suggested by Bamford, Elliott and Hanby (1956). It has to be realised that some early work may have been on polymers of doubtful constitution and low molecular weight, and attempts to deduce the conformation were premature until the early 1950's. Experimental work had mainly been within various schools of surface chemistry by people who had come to accept the traditional view of an extended polypeptide at the interface, and who tended to rely on the standard methods of surface chemistry. Their techniques were frequently elaborate, though simple in principle, and fundamentally unchanged for many years. Their

measurements of surface pressure and surface potential as a function of monolayer area, and measurements of surface viscosity, had become a rather isolated branch of science.

Geographical isolation may also have contributed to the perpetuation of ideas that were in need of reconsideration, since a high proportion of the early publications in this field are from the laboratories of Isemura and co-workers and later Yamashita, in Japan. Despite the comments of Bamford et al. (1956) others active in this field have accepted Japanese work largely at face value (Crisp, 1958; Joly, 1964; Llopis, 1968). This covers a wide range of polymers and copolymers and a review is not attempted here, but a number of their papers are referred to in the papers in this section.

It is also surprising that in view of the increasing attention being given to interfaces in biology, and progress in the use of computer methods in predicting molecular conformations, little theoretical consideration has been given to surface structures. Probably one reason is that theoretically they are much more complex than structures in the solid state or solution. All the standard conformations are derived by operating on the asymmetric unit with a screw translation, so that all residues are equivalent. If any such structure is located at for example, an air-water interface, some

residues have their side chains directed into the air and others into the water, and they cease to be equivalent. It is then unlikely that if the side chain is flexible it will be in the same conformation on the two sides of the interface and possibly the backbone conformation may also be modified.

These considerations provoke a number of questions. If the side chains are hydrophobic, might the molecule adopt a conformation that does not conform to the usual criteria? Is it possible that the peptide groups all hydrogen bond to water? Are alternations of cis and trans peptide groups a possibility? What would be the consequences of various alternations of hydrophobic and hydrophilic residues? Since rod-like molecules such as the α-helix would be constrained to pack in parallel groups, would this give rise to additional stability?

Answers to these questions might be obtained by a purely theoretical approach, but often in this field theoretical advances are stimulated by experimental observations and tend to be retrospective in character. In any case it was clear that an experimental study of interfacial structures would be potentially interesting and important, provided that the obvious difficulties in a study of the structure of a monomolecular layer could be overcome.

To make further progress it was considered that since the standard techniques of surface chemistry did not produce information that was free from ambiguity one had to combine them with new methods, used preferably on the monolayer *in situ*, but if necessary by removing it from the surface and using more powerful indirect methods. This latter approach is obviously one that has to be used with great caution, and on many systems it might be quite unjustifiable and wholly misleading. One has to consider carefully the possible consequences to the polymer in removing it from the water surface and placing it in some other environment. That such experiments hold out some hope of success depends on the fact that synthetic polypeptides in the solid state frequently show evidence of their past history - they seldom immediately come (if ever) into their most stable state. This type of experiment has even produced significant results in relation to conformations in solution, where early work showed that it was possible to relate them to structures in the solid state (Robinson and Bott, 1951). But to place too much weight on the results of such experiments alone is as mistaken as to ignore their possibilities and refuse to consider their merits.

Clearly in this field a combined approach using all available methods is essential. Nevertheless in studies of monolayer structures it is difficult to achieve anything like the degree of reliability we

associate with that attainable with work on the solid state or solutions. Even when a combination of method leads to a simple self-consistent picture, an element of doubt remains. Each individual approach seldom fails however to provide new information about a system, even though at the outset an experiment might be considered to be of no more than of confirmatory value. When new and unexpected information is obtained which nevertheless fits into place one has grounds for confidence; if it does not, it may or may not be of significance, since it too usually has an element of unreliability. But to pass it over might be disasterous; one then has to exercise a combination of objectivity and insight.

Paper 13. It is possible that one reason the surface chemistry of these polymers has been neglected is that too many individuals have had unfortunate experiences with Langmuir troughs in their early days, or their education in this direction has been neglected for want of a simple apparatus. The development of the film balance described greatly expedited this work.

The standard type of film balance, with a float and torsion balance, is far from ideal. Torsion devices tend to be delicate and can usually be replaced with a more robust and simple flexure device without real loss of sensitivity. The time to set up the standard apparatus is

considerable so that contamination of the water surface builds up or is accidentally introduced. Sealing the float to the edge of the trough requires skill and practice if leaks are to be avoided. Its lack of rigidity makes it difficult to remove a collapsed monolayer at the end of an experiment (for spectroscopic examination). These considerations led to the development of the simple flexure device described. It takes about a minute to set up from the time the trough surface has been swept clean, it is normally free from leaks and requires no special skills in its use.

(Flexure devices frequently depart from simple theory rather seriously and I therefore developed this in collaboration with Davies who carried out calculations on deflections from point and distributed loads for various configurations of the strip. These showed that it could be made an absolute method by calibrating it with a point load. However, in practice it is more convenient to calibrate it with a "piston oil".)

Paper 14. It proved surprisingly easy to collapse and remove a monolayer from the water surface and obtain its infrared spectrum in the dry state. The monolayer areas were consistent with α-helical but not β-conformations. The deuterium exchange experiments were designed to exclude the possibility of peptide-water hydrogen bonding, but in view of the time taken to spread and remove a monolayer it would not have been surprising if even α-helical structures had been found to exchange completely. Control experiments on exchange in nylon copolymers (where peptide-water hydrogen bonds almost certainly exist) showed exchange too fast to observe (paper 20).

Paper 15. Since both monolayer studies and those on collapsed films pointed to the presence of the α-helix, it was then possible to propose an explanation for the significance of the plateau in the pressure-area curve. I had earlier tended to regard this feature in published Japanese work as no more than instability of the monolayer under pressure, while they appeared to take the view it was a conformational change. However the remarkable steadyness of the surface potential during the transition in a number of polymers, and the reproducibility of the plateau height, were additional evidence against any conformational change or a simple collapse of the monolayer, and showed that it was a characteristic of

the polymer. Moreover after the transition the film was less compressible, as if it were becoming stronger rather than simply buckling.

Paper 16. Collectively the preliminary results were good evidence for the presence of the α-helix in the monolayers of a variety of polymers and considerable time spent developing the techniques proved profitable. The collapsed films had infrared spectra agreeing in detail with specimens in the α-helical conformation, and no more than confirmation of the presence of the α-helix might have been expected from electron diffraction studies. However, two unexpected features appeared, during collapse of the monolayer the molecules orientated quite considerably, and more surprisingly in the particular case of poly (γ-methyl-L-glutamate), a 'single crystal' type of diffraction photo was obtained. There are a number of features of the electron diffraction work which would repay further study with a suitable instrument.

Loeb and Baier (1968) working independently on poly (γ-methyl-L-glutamate) obtained agreement with the pressure-area curve I had reported (Paper 15) but did not measure the surface potential or recognise its significance. Following Japanese work, they found a low area (10 $Å^2$/residue) when chloroform-pyridine mixtures were used to spread the monolayer. Despite my comments on the Japanese work (paper 15) (based on the view that since pyridine is not a solvent

for the polymer and is miscible with water it was best avoided) they regarded the correlation of a short or absent plateau coupled with an increasing β-component in the infrared spectrum as significant, seeking to relate it to the same area observed after the plateau, when a monolayer was spread from solution in chloroform.

From their results one can conclude that the more of the β-conformation present in the monolayer the shorter the length of the plateau, but this does not take one any further forward in understanding the formation of the plateau, except to suggest that it is not a direct property of the β-conformation. My interpretation of their results is that the β-component arises, perhaps as a separate phase, essentially as a result of precipitation during the initial spreading; from its low area it is clearly not a true monomolecular layer. As Loeb and Baier remark, "Mixing of water with the spreading solution may have a profound influence on the polypeptide structures formed".

Loeb and Baier were however able to obtain a spectrum from a single deposited layer using the multiple internal reflection technique, so that they did not have to collapse a film through the transition region as in the method I used. Similar spectra were obtained from films deposited above or below the transition pressure and they considered that their work showed that the conformation was unaltered through the transition. If they were correct in this then they

disregarded the electon diffraction results referred to in Paper 15, in supposing a random coil in the monolayer to be a possibility, and they do not seek to explain how a random coil could give rise to such a sharp transition.

Further evidence for the basic correctness of my interpretation comes from the calculation of the work of cohesion of the polymer derived from the height of the plateau and the angle of contact of water at the polymer surface. The value obtained for poly(γ-methyl-L-glutamate) of 91 erg/cm^2, leads to 45.5 erg/cm^2 for the free surface energy of the polymer-vapour interface. This agrees better than might be expected with the range 40 to 50 dyn/cm subsequently found (Baier and Zisman, 1970) for the critical surface tension - a quantity considered closely related conceptually to the surface free energy (Baier, Shafrin & Zisman, 1969). Measurements of the critical surface tension on other polypeptides would be of great interest in this connection. It might also be possible to show a correlation of the values for the free energy and the ultimate strength of fibres formed from polymers in the same conformation.

Papers 17, 18 & 19.

In general I have never been in favour of studying intensively one particular polymer in order to understand the general rules that might be expected to apply to a whole class of materials. However, once a general pattern of behaviour has been established, it is obviously relevant to look at individual polymers that might prove exceptional in some way. Not all candidate materials prove suitable. Poly-glycine for instance does not appear to form a true monolayer, others such as poly-L-tyrosine and poly-DL-phenyl-alanine do not have plateaux in the surface pressure curves (Yamashita and Isemura, 1962) for reasons not altogether clear. Two factors at least determine whether there is a flat plateau; firstly the side chain should exceed a certain length, as pointed out by Crisp (1958), secondly from the type of exceptions mentioned above it appears that it should be flexible. In addition the adhesion between the monolayer and subsolution must be high enough to resist the tendency for the polymer to collapse on account of the free energy of the polymer-vapour surface - otherwise the monolayer is unstable and the plateau can be considered to occur at a negative pressure.

Two polymers became available that the foregoing considerations suggested would be of great interest. Poly (β-benzyl-L-aspartate),

unusual in that the α-helix in it is normally left handed (one could have said always left handed prior to this work), and compared with the right handed helix relatively unstable; it also forms an ω-helix with a four-fold screw axis. The other was poly (ε -benzyloxycarbonyl-L-lysine), chosen for the length and flexibility of its side chain and because it has a peptide group in the side chain as well as the backbone.

Both these polymers followed the general pattern of the behaviour of the earlier work, but proved to have additional features of great interest that were exceptional, though some doubts have been expressed, not surprisingly, in connection with the pressure-area curve of poly(ε -benzyloxycarbonyl-L-lysine). Gaines for example referred to the 'somewhat structured force-area curves' (A.C.S. meeting in Chicago 1970, in relation to Paper 24 in preprint form), and in his book (Gaines, 1966) he points out that well defined poly-molecular films, two or three molecules thick, are not thermodynamically stable, - that is at pressures above that of the first plateau in the case of the polypeptides. It is I think reasonable that a compressed polypeptide monolayer be regarded as a 'solid' where small departures from thermodynamic equilibrium are frequent, and indeed add interest, for example, as shown by the experiments described in Paper 17.

I have therefore regarded multilayer formation in terms of the deformation of an almost perfect plastic in the form of a thin plate compressed 'edge on'. To understand the build up of the thickness of the film one then has to suppose that something analogous to the propagation of a dislocation in a crystal occurs through the thickness of the film, so that molecules are transported to the surface. Clearly the physical picture is far from complete, particularly in regard to the role of the side chain, and more evidence is required to enable a full description of the process to be developed.

While the transition from monolayer to multilayer is of considerable interest in its own right, it is also relevant to the formation and stability of tertiary structures in proteins, where as in the formation of a bilayer, side chain-water adhesion is balanced against side chain-side chain interactions in the interior of the molecule.

In conclusion it appears that the surface chemistry of the synthetic polypeptides I have studied is now generally accepted, both in relation to the methods employed and the interpretation of the results (Loeb, 1970; Caspers, Ruysschaert & Jaffe, 1970; Miller, 1971). Loeb and Baier (1970) however still appear reluctant to accept the α-helix in the monolayer as a working hypothesis, though none of their results exclude it. Their work has been mainly on one

polymer, poly(γ-methyl-L-glutamate), and their infrared spectra rest on the Amide I and II bands only. They point out the limitations of model building and do not appear to accept that it can be used to exclude possible conformations. While quite properly concerned with the limitations of individual methods they do not attempt, as I have done, to develop a simple self-consistent picture that applies not only to one polymer but to a wide range of others.

SECTION VI (Papers 20, 21 & 22)

The Structure of proteins at interfaces and cell surfaces.

In parallel with the studies of the synthetic polymers it was obviously desirable to try to relate the work to the surface chemistry of proteins and the structure of biological membranes. The main point was to show that experimental work in this field and theories of membrane structure required reconsideration in the light of improvements in our knowledge of protein structure, surface chemistry and the experimental methods that might be applied. In particular it was hoped that surface chemists and biologists concerned with membrane structures would recognise that the widespread acceptance of the presence of the β-conformation was largely a historical accident and that other structures such as the α-helix were equally possible, and these papers aimed to demonstrate the types of experimental methods that could be applied. In this respect collaboration with Maddy, who was particularly concerned with the biochemical properties of membrane proteins, was timely and valuable.

Paper 21 brought a quick reaction from Kavanau (see Paper 22) but the validity of our views has been generally recognised. Our work was the first to show that infrared spectoscopy and optical rotatory dispersion could be applied to problems of membrane structure

and it was quickly followed up by papers by Wallach and Zahler (1966) and Lenard and Singler (1966). The main extensions to our work have been to apply the methods to other membrane systems and to include studies of circular dichroism. In so doing considerable confusion has been generated over the validity and meaning of the circular dichroism data (see for example Choules and Bjorklund, 1970).

It is now recognised that the α-helix may be present as a structural component of membranes together with other conformations, that the proportions are capable of experimental determination and probably vary from one system to another. Some, however, still appear reluctant to accept the possible consequences of this work. Pethica and Cambrai (1970) for example comment in relation to the optical rotatory dispersion work, "Used particularly by Wallach, this method has shown beyond doubt that, in certain membranes proteins are helical. While proteins can exist in this form at interfaces, notably in the air-water system, it is difficult to see how an α-helix can be associated with lipids as in the Danielli model", (my translation).

Thus the debate continues, and I think that the approach developed in this work has produced an important reconsideration of our views and methods. However, I feel that it is doubtful whether further results on the same lines will produce information of major significance, unless it is supplemented by other methods.

SECTION VII (Papers 23 and 24)

Interactions of synthetic polypeptides with water.

The structure and properties of water in relation to proteins has been the subject of debate and numerous experiments over many years. Detailed interpretation of much early work was hampered by the inability to distinguish between interactions of the water with the backbone and with charged groups on the side chains, quite apart from the unknown complexities of many proteins. Use of a synthetic polypeptide with uncharged side chains is therefore rather obvious, were it not that these are nearly all insoluble in water and relatively hydrophobic. However, the measurements of contact angles against water (Paper 16) and the fact that poly D, L-alanine is water soluble, suggested that it should be possible to observe adsorption of water from the vapour phase on the less hydrophobic polymers by infrared spectroscopy, particularly as this had already been seen in spectra in the near infrared (Paper 1) at 5150 cm^{-1}. The results reported (Paper 23) are very significant. While they might have been obtained at any time in the last fifteen or so years by almost anyone working in this field, the method of producing fairly thick oriented specimens that was used is important, since other methods almost always lead to some extended β-conformation being produced, which would greatly complicate

interpretation of the results. The absence of this structure is shown by the electron diffraction observations (Paper 16) as well as by polarised infrared spectroscopy. The type of spectrometer used for this work was not ideal, since a higher dispersion than that available with the NaCl prism would be valuable. It was therefore decided to publish a short note in the anticipation that others with more suitable instruments would follow it up.

It did, however, seem of interest to combine a discussion of the origins of the surface potential in monolayers with further observations of the polarized infrared spectra of water, since it appeared that there might be a connection between them. Surface potential measurements relate to observations of static polarization effects whereas in the spectroscopic studies one is concerned with polarization using infrared frequencies. Since in both instances it appeared that water molecules were in some way orientated this approach seemed reasonable and, as the results show, there may be a correlation. The basic problems of understanding the origins of the surface potential have existed for many years and further experiments along the same lines may resolve some of the main issues, as well as providing new information concerning water-polymer interactions.

Paper 24 was to be presented at a meeting of the American Chemical Society and the opportunity was therefore taken to correlate

and extend the surface chemistry of the polypeptides, and present it to an informed American audience. Their reception of it and discussions I had elsewhere in the U.S.A. were stimulating and valuable. In this respect I would like to record my appreciation for helpful discussions with Dr. R.E. Baier (Cornell Aeronautical Laboratory) and Professor R. Good (Department of Chemical Engineering, State University of New York at Buffalo) in Buffalo, and Dr. G.I. Loeb and Dr. W.A. Zisman (U.S. Naval Research Laboratory) in Washington D.C.

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The near infra-red absorption spectra of natural and synthetic fibres*

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The near infra-red region may be used to obtain spectra with polarized radiation of natural and synthetic fibres. This region has considerable experimental advantages and the spectra of poly-*L*-alanine, polyglycine, wool, silks and commercial regenerated protein fibres are shown and discussed. Besides supplementing other types of observation, information may be obtained on the orientation and molecular configuration not always given by other methods.

The infra-red absorption spectrum is capable of providing considerable information regarding the composition, degree of molecular orientation and in some cases amount of crystallinity of fibres. In the range 3 to 10 μ wavelengths, the thickness of the specimen necessary to obtain the proper strength of absorption is restricted; in the case of protein fibres, for example, the specimen should usually be not more than 5 μ thick. Thicker specimens have to be reduced either with a microtome or by grinding. The width of the specimen is also important. In order that the image of the specimen shall fill a 0·1 mm wide spectrometer slit a circular fibre 5 μ in diameter has to be magnified at least 20 times and in actual practice considerably higher magnification is desirable. The design of a suitable instrument presents considerable problems. Moreover, in order to fill the aperture of the spectrometer it is necessary to use a wide cone of radiation from the specimen. When used with polarized radiation the effect of this is to lower the dichroism and corrections are difficult to apply. This is therefore a region which is not at all suitable for the rapid examination of fibres and raises considerable experimental difficulties.

In the region 1–2·5 μ the bands are either overtones or combinations of the fundamental vibrations, with much lower absorption coefficients so that a specimen may be about 0·2 mm in diameter. These may be used with a reflecting microscope of about ×5 magnification. Thinner specimens can be examined by tying a number of fibres together in a small bundle and immersing them in a suitable transparent liquid of about the same refractive index, to reduce the amount of scattered radiation.

From an experimental standpoint this region has other considerable advantages. The source may be a tungsten filament lamp in a glass envelope. The spectrometer prism can be of second quality fused quartz which has a high dispersion and is quite inexpensive. A lead sulphide cell may be used as the detector with an 800 c/s amplifier. This avoids many of the troubles commonly associated with low-frequency amplifiers for thermocouples. Cells for liquids can be made from glass, and since the wavelength range is fairly close to the energy peak of the source, trouble from stray radiation is seldom serious. These advantages are considerable and make the exploration of this region for the examination of fibres well worth while.

The most serious disadvantage of this region is that at our present state of knowledge it is hardly ever possible to be certain of the origin of all the bands, which are often superposed one on another so that quantitative measurements on them are less reliable. An empirical approach can, however, yield considerable information in certain cases and the difficulties mentioned are not entirely avoided when working in the fundamental region.

The type of information which can be derived from a study of the spectra in the 1–2·5 μ range is best illustrated by the examples which follow. Most of these refer to protein fibres or synthetic polypeptides. While these appear particularly interesting there is no reason *a priori* to assume that a similar wide investigation of other types of fibre might not also provide information of equal interest.

One of the most useful applications of this region is measurements on the dichroism of bands in order to estimate the degree of molecular orientation in the fibre. In this respect the measurement is usually taken over both the crystalline and amorphous regions since normally these are undifferentiated in the spectra. Infra-red observations therefore are complementary to an X-ray examination which reveals the orientation only of the crystalline regions and the two may be profitably combined.

Experimental Methods

Many of the features of apparatus used to obtain the results which follow have already been mentioned. We have used either a Perkin–Elmer single-beam spectrometer with a lithium fluoride prism or a spectrometer constructed by ourselves with a fused quartz prism. This instrument has two spherical mirrors arranged in the manner shown by Tetlow, McAuslan, Brinley and Price[1] after the method recommended by Czerny and Turner[2] and avoids the use of an off-axis parabolic mirror. Both instruments are fitted with reflecting microscopes giving ×5 magnification and the specimen is moved in and out of the beam at two-second intervals. In this way the spectrum of the specimen and the incident beam are obtained almost simultaneously and errors caused by long period fluctuations in the apparatus are minimized. This method of recording contributes greatly to the accuracy of the result and is of great assistance in observing small differences in otherwise similar spectra such as are produced when the dichroism is low. The polarizer used has been described by Elliott, Ambrose and Temple[3,4] and the recorder is a modification of that described by Elliott and Ambrose.[5]

When measurements are being made on a large number of fibres which are optically inhomogeneous it is often impossible to reduce the scatter by means of an immersion liquid to a sufficiently small amount for it to be comparable with the true absorption. In such cases the reference beam may be defocused by placing a piece of glass over the part of the cell through which the reference beam passes. If I is the

* This article was read at a meeting of the Industrial Spectroscopy Group of The Institute of Physics on 29 April, 1954.

transmitted intensity, I_0 the incident intensity, a and s the absorption and scattering coefficients, then

$$I = I_0 \exp[-(a + s)d]$$

where d is the thickness of the specimen. In general both a and s depend on the direction of the electric vector relative to the fibre axis. If the reference beam is reduced in intensity by a fraction k we obtain

$$\log(kI_0/I) = (a + s)d$$

$\text{Log}_{10}(I_0/I)$ is the optical density and is measured from the spectrometer record. Since both s and k vary only slowly with wavelength compared with a, the absorption bands appear superposed on an arbitrary, even background. This may be estimated if there is a region in which the fibre is known to be transparent and subtracted if necessary. For many purposes, however, all that is required is to compare the shape and relative intensities of bands and in this case the absolute zero does not need to be estimated.

The dichroic ratio of a band in a fibre is measured by the ratio of the absorption coefficients parallel and perpendicular to the fibre axis. It depends on the angle α of the transition moment giving rise to the absorption, relative to the molecular axis and the orientation of the molecules relative to the fibre axis. The dependence of the dichroic ratio on α and on the average angle of orientation has been calculated by Elliott, Ambrose and Temple[6] for $\alpha = 0°$ and 90°. Qualitative comparisons between fibres of the same type can be made even if α is unknown since dichroism cannot arise without molecular orientation. It does not follow, however, that since a fibre is not dichroic that there is not a regular molecular structure, as for example in some biological structures where layers of fibres may develop in a criss-cross pattern.

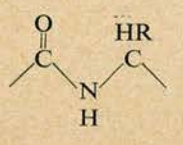
GENERAL FEATURES OF OVERTONE SPECTRA

Most fibres contain some water which gives rise to a strong band at about 5150 cm^{-1}. Quite small amounts are detectable so that the water uptake of textile fibres can be measured quantitatively.[7] Examination of the infra-red spectrum is probably the most reliable way of finding out whether a fibre has any residual water in it after drying.

In the range 4350–4450 cm^{-1} there are often quite sharp bands which are combinations of CH stretching and deformation modes. At lower wave numbers, 4350–3900 cm^{-1}, while often there are a number of resolvable peaks, their assignment is difficult since they appear to arise from combinations with skeletal modes.

Fibrous proteins of the keratin type have long been known to exist in two forms; in the α-form the polypeptide chain is folded or coiled in some way whereas in the β-form the chains are much more nearly extended (Astbury and Street[9]). More recently, synthetic polypeptides have been shown to occur in two forms which have many of the characteristics of α- and β-keratin (Bamford, Hanby and Happey[10]; Ambrose and Elliott[11]). These forms are also referred to as α and β, though it is recognized that the polypeptide chain configurations are not identical with the corresponding forms of the fibrous proteins.

The repeating unit of the polypeptide chain is



where R represents a side chain. In the synthetic polypeptides polyalanine and polyglycine R is CH_3 and H respectively and natural proteins are characterized by a wide variety of side-chains. Silks are particularly simple proteins consisting mainly of alanine and glycine. It has been shown by Ambrose and Elliott[12] that the two forms α and β may be distinguished by two absorption bands of the polypeptide chain in the overtone region. The first of these is a band at 4850 cm^{-1} which has high parallel dichroism in extended (β) polypeptides and perpendicular in folded (α) polypeptide chains. This is probably the same band as that observed in nylon by Glatt and Ellis[13] at 4883 cm^{-1} and is assigned by them to a combination of NH stretching and in-plane deformation modes. The second band is at about 4520 cm^{-1} in the β-form and at about 4600 cm^{-1} in α polypeptides and proteins. The band at 4520 cm^{-1} shows parallel dichroism comparable with the NH combination, but dichroism is usually not observed in oriented α-fibres in the 4600 cm^{-1} band. Reasons have been given for assigning this band to a CO combination mode[12] but the fundamental frequencies have never been identified. Recent experiments on the deuteration of synthetic polypeptides have shown that on replacing the amide hydrogen by deuterium this band disappears at the same rate as the NH combination band. It therefore seems unlikely that it is a simple CO combination mode and probably directly involves the NH group. Price and Fraser[14] have suggested that a band at 1270 cm^{-1} in the spectrum of α-proteins is predominantly a C–N vibration and it seems possible that this couples with the NH stretching band at 3300 cm^{-1} to give the band at 4600 cm^{-1}. This is supported by the disappearance of this band and the development of a band at 1220 cm^{-1} in poly-*L*-alanine when it undergoes the α- to β-transformation, which would be consistent with the observed frequency shift of the combination. This will be discussed more fully elsewhere; whatever the precise origin of this band, its empirical correlation with the α- and β-configurations is well established and is consistent with infra-red observations on the fundamental CO and NH modes and X-ray diffraction results. These results have been applied by Elliott to an examination of the stretching of hair[15] and by Ambrose and Elliott [16,17] to globular proteins.

There is a remarkable agreement between the main frequencies of the globular proteins and the synthetic α-polypeptides and the frequency shifts on denaturation corresponding to a partial transition to the β-form. This cannot, however, be taken to imply that the α-configuration is necessarily the same in globular proteins as in synthetic polypeptides with inert side chains, where a helical structure is consistent with most of the experimental results. As yet no crystalline globular protein has been found to give high infra-red dichroism and X-ray studies have not so far given an unambiguous result. This must be remembered when comparing the spectra of synthetic and polypeptide fibres with regenerated protein fibres.

EXAMPLES OF INFRA-RED SPECTRA OF FIBRES

Poly-L-alanine.

α-form: Fibres of poly-*L*-alanine which contain a small amount of dichloracetic acid may be oriented by stretching, and are then found to be predominantly in the α-form, though a small amount of the oriented and crystalline β-form is also produced. The fibres may be suitably conditioned by soaking in a solution containing 12 parts of dichloracetic acid in 100 parts of carbon tetrachloride (by volume), and then

drying in air for about 15 min in order to allow the carbon tetrachloride to evaporate. Cold drawing is accompanied by very pronounced "necking," and it is noteworthy that even quite irregular fibres may be successfully drawn in this way. The extension possible is fairly constant, about 170%. The dichloracetic acid may be removed by heating or by washing, for example in ether or carbon tetrachloride. Removal by heat produces a very crystalline specimen, from which a very well-ordered X-ray diagram may be obtained [see Bamford, Brown, Elliott, Hanby and Trotter[18] Fig. 1(*a*)]. Washing out the acid produces a much less crystalline fibre, but the infra-red spectrum appears to be the same in both cases, except for the fact that the water band at approximately 5250 cm^{-1} is much reduced in the spectrum of the heated specimen.

The spectrum of a heated specimen is shown in Fig. 1.

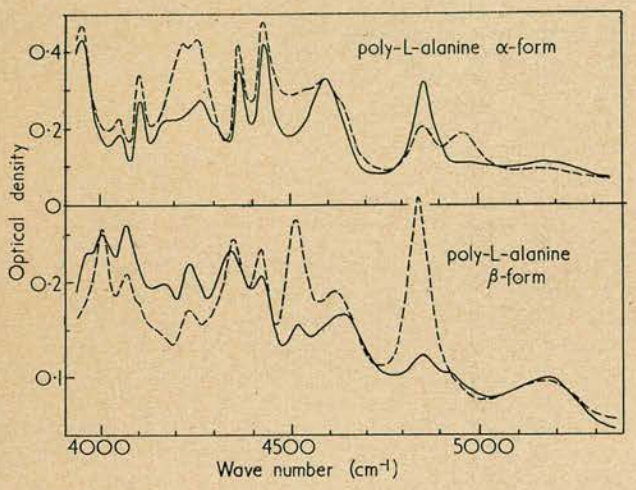


Fig. 1. Spectra of oriented fibres of α (folded) and β (extended) poly-*L*-alanine observed with polarized infra-red radiation

Full line—E vector perpendicular to fibre axis.
Broken line—E vector parallel to fibre axis.

β-form: Cold drawing of an air dried, poly-*L*-alanine fibre results in the production of an oriented β-fraction, with a roughly equal amount of slightly oriented α-material. Similar results are obtained if a water-soaked fibre is drawn at room temperature. If, however, the stretching is carried out in steam, a greater proportion of the polymer is extended into the β-form and high crystallinity and orientation of this form is obtained [Bamford and others[18] Fig. 1(*b*)]. The spectrum of a poly-*L*-alanine fibre stretched in steam is shown in Fig. 1.

The fact that the predominance of (respectively) α- and β-forms of the polymer has been convincingly demonstrated by the methods of X-ray diffraction greatly strengthens the validity of the interpretation of the spectra shown in Fig. 1. In particular, the association of the parallel band at 4510 cm^{-1} with a β-configuration and of the non-dichroic band at 4600 cm^{-1} with the α-form are confirmed. In polyalanine, there is also a small frequency change in the combination band from 4845 cm^{-1} (β) to 4860 cm^{-1} (α), though this shift is too small to be of value for diagnostic purposes.

The bands at 4365 cm^{-1} and 4425 cm^{-1} in α-poly-*L*-alanine, and corresponding bands at a slightly lower wave number in the β-form are almost certainly CH_3 combinations of stretching and deformation modes, as may be seen by comparing them with bands in the spectrum of methylene chloride.[19] Some of the bands at lower wave numbers show quite high dichroism, but as yet nothing is known of their origin. The very highly dichroic band at 4965 cm^{-1}, noted by Glatt and Ellis[20] as appearing in some nylon specimens, is very prominent in the spectrum of α-poly-*L*-alanine, but hardly to be seen in the β-form. Like the band at approximately 4850 cm^{-1}, its dichroism changes with change of configuration from α to β. If, as seems likely, the 4965 cm^{-1} band is associated with crystal lattice modes, it is nevertheless not possible to correlate its strength with the "crystallinity" of a polymer as deduced from the X-ray diffraction diagram. In support of this statement, it may be mentioned that oriented α-poly-*L*-alanine which has not been heated (see above) shows the band quite strongly: it is also prominent in poly-*DL*-leucine.[8] However, the appearance of an X-ray diagram depends to a great extent on crystallite size (and, of course, orientation) and possibly infra-red bands would be produced by much smaller crystal regions than would be needed to produce sharp X-ray reflexions.

Polyglycine.

The specimen of polyglycine (Fig. 2) was not of sufficiently high molecular weight to enable it to be oriented and was

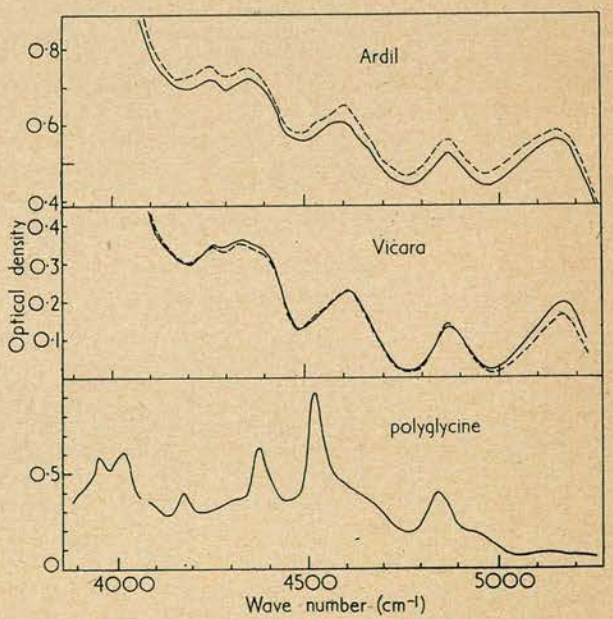


Fig. 2. Spectra of polyglycine (unoriented) and of commercial regenerated protein fibres

Full and broken lines as in Fig. 1.

prepared by casting a film from trifluoracetic acid. It is probable that it contains a proportion of small molecules. The band at 4520 cm^{-1} shows the β-configuration is present and is unusually sharp. The shoulder extending to 4700 cm^{-1} extends too far for it to be the α-component of this band and is probably a consequence of the small peptides. Possibly the shoulder on the NH combination band has the same origin.

By analogy with polythene two bands were expected arising from the CH_2 group but there is apparently only one band at 4375 cm^{-1}. The symmetrical CH_2 stretching mode is considerably weaker than the anti-symmetrical mode so that

the second band may be the very weak one at 4300 cm^{-1}. This would be consistent with the fundamental CH_2 stretching and deformation frequencies.

Wool and regenerated protein fibres.

The main difference between silks and the proteins present in wool and regenerated protein fibres is in the much larger variety of side-chains attached to the polypeptide chain in the latter in appreciable quantities. These may form cross-links of various types and favour the formation of secondary folds. It is therefore interesting to note that while the absorption bands of their polypeptide chains resemble silk and synthetic polypeptides there is an almost complete absence of dichroism (Figs. 2 and 3). The band at 4600 cm^{-1} shows that in all cases the α-configuration is predominant.

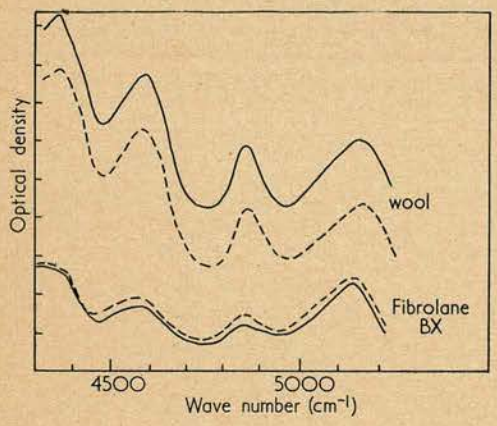


Fig. 3. Spectra of wool and of regenerated commercial casein fibre

Full and broken lines as in Fig. 1.

There is, however, in many cases an asymmetry caused by a slight shoulder towards 4520 cm^{-1}. This is usually more noticeable in the parallel position of the polarizer and indicates a certain amount of slightly oriented β-material. In the case of Fibrolane (a casein protein[21]) the orientation of the β-material is just sufficient to give detectable parallel dichroism in the NH combination band. Vicara (made from zein) appears to have rather less β-material than the other regenerated protein fibres and its spectrum most closely resembles that of wool.

The high water-uptake of these fibres is shown by the strong band at 5150 cm^{-1} but direct comparison is not possible since the measurements were not carried out under conditions of controlled humidity. Below 4400 cm^{-1} these fibres all have ill-defined bands without any distinctive features which is a consequence of the wide variety of side chains present.

Silk.

Some years ago Bath and Ellis[22] examined the spectrum of silk fibres in the overtone region, using polarized radiation. They reported marked dichroism, and refer to a perpendicular band at 1·93 μ (5180 cm^{-1}) which they ascribed to the second overtone of the C=O stretching mode. This is in the region of the band now ascribed to water, which, however, is not dichroic. We have examined silk gut which was dried at 100° C for 24 h and found that the 5150 cm^{-1} disappeared completely, with no appearance of any band which might have been overlaid by the water band. Now Bath and Ellis dried their specimens in an oven for several days at 110–115° C, and it therefore seems likely that what they observed was a band due to some product of oxidation or decomposition of silk (which is not a very heat-stable material). The thicker silk gut which we used would have very much less surface exposed to the air than the cocoon fibres used by Bath and Ellis. Their observations on the first overtone of the NH stretching mode are interesting, for this band was found to have three components showing perpendicular dichroism, which is in agreement with silk fibroin having an extended configuration. The first overtone of the NH stretching mode lies outside the region which we have examined.

The examination of silks provides a good example of the value of infra-red observations in the overtone region. Although silk gut can be sectioned to allow observations in the region of fundamental absorption bands (Ambrose and Elliott[12]), it cannot be assumed *a priori* that the structure of silk gut and cocoon silk (from the same species of moth) are identical, though the X-ray diffraction patterns suggest this.

Fig. 4 gives the spectra of several cocoon silks. Cocoon silk of Bombyx mori gives a spectrum which is indistinguishable from that of commercial silk gut. The spectra of all

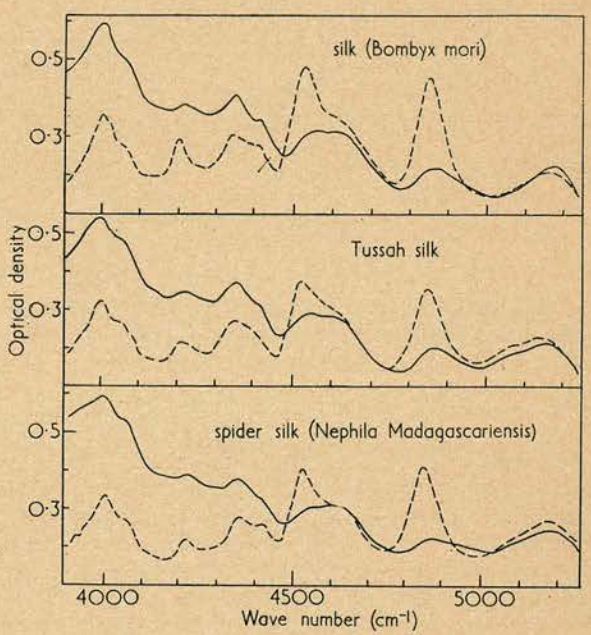


Fig. 4. Spectra of silk fibres

Full and broken lines as in Fig. 1.

three silks are notably similar to that of β-poly-*L*-alanine. In all the silks, the non-dichroic band at approximately 4600 cm^{-1} is quite prominent, and shows, in our opinion, that an appreciable fraction of the polypeptide chains in silk are in a folded configuration. There are, however, no reflexions in the X-ray diagram of the silks hitherto examined which resemble those of α-poly-*L*-alanine, and it would appear that the folded chains in silk are too amorphous to give a recognizable reflexion. Water-soluble silk made from solution in aqueous lithium bromide has a band at 4600 cm^{-1} (Toms and Elliott[23]) and gives an almost completely amorphous X-ray diagram (Ambrose, Bamford, Elliott and Hanby[24]).

The apparent high dichroism at the low-frequency end of the spectrum is not real. Because silk fibres are very thin, it is necessary to use a large number in order to obtain sufficiently strong absorption. Reflexion losses are therefore considerable, and these change with the direction of the electric vector, giving rise to what is known as "form dichroism." The effect can be reduced at any chosen wavelength by matching the refractive index of the immersion liquid suitably, but in general the effect will appear at other wavelengths, on account of dispersion. In judging dichroism, therefore, the height of a band above the local background is the best measure of intensity.

CONCLUSION

While it is clear from the above account that there are limitations to the information which can be obtained from the examination of infra-red spectra in the overtone region, it should be apparent that the method is capable of supplementing other kinds of investigation. In protein and polypeptide fibres, especially, information on chain configuration can be obtained which has hitherto not been given by other methods. There is, moreover, good reason for thinking that with the accumulation of information concerning the origin of the absorption bands more will be deduced from overtone spectra.

The small amount of extended polypeptide in the regenerated proteins seems to be well established and (though this is speculative) may mean that they are still essentially corpuscular in character. Another striking result is the fairly constant proportion of folded configuration present in the different silks examined, and in steam-stretched poly-*L*-alanine.

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SOME OBSERVATIONS ON THE INFRA-RED SPECTRUM OF MUSCLE

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INTRODUCTION

In the near infra-red region between 4500 and 4900 cm.$^{-1}$ there are two bands caused by absorption of radiation by the peptide groups in proteins and synthetic polypeptides. By examining these bands with polarized radiation and correlating the results with X-ray diffraction photographs and absorption bands in other regions of the infra-red, it is possible to relate the frequencies and dichroism to the molecular configuration and orientation. Results obtained in this way have been applied by Ambrose & Elliott (1950, 1951*b*) to globular proteins and by Elliott (1952*a*) to an examination of the stretching of hair. These observations have recently been further confirmed by the preparation and X-ray examination of well-oriented and highly crystalline forms of both α- and β-poly-L-alanine (Bamford, Brown, Elliott, Hanby & Trotter, 1954), and their near infra-red spectrum has been obtained by Elliott, Hanby & Malcolm (1954). It is shown here that if the spectra of these model compounds are compared with the spectrum of frog sartorius muscle, limited but quite definite conclusions can be drawn concerning the molecular configuration and orientation in muscle.

EXPERIMENTAL PROCEDURE

On account of the weak absorption of the peptide group in the range 4500–4900 cm.$^{-1}$ compared with its strong absorption in the 3300 cm.$^{-1}$ and 1500–1700 cm.$^{-1}$ regions, it is possible to pass the radiation through a whole frog sartorius muscle. The strong absorption of water makes measurements difficult, but it is sufficiently transparent in a thickness of about 1 mm. for the absorption spectrum of live muscle in Ringer's fluid to be obtained, provided that the muscle is slightly stretched to reduce the scatter of radiation.

The instrument used was a Perkin-Elmer Spectrometer Model A with a lithium fluoride prism. The main details of the techniques used have been described elsewhere (Elliott *et al.* 1954); however, on account of the strong absorption of water some refinements were necessary. Scattered non-monochromatic radiation from the spectrometer is seldom troublesome in

this region, but the transparency of water at shorter wave-lengths, compared with the range under examination, necessitated the use of filters to remove stray radiation of shorter wave-lengths. For this purpose pure germanium is very suitable since it absorbs strongly above 5000 cm.$^{-1}$

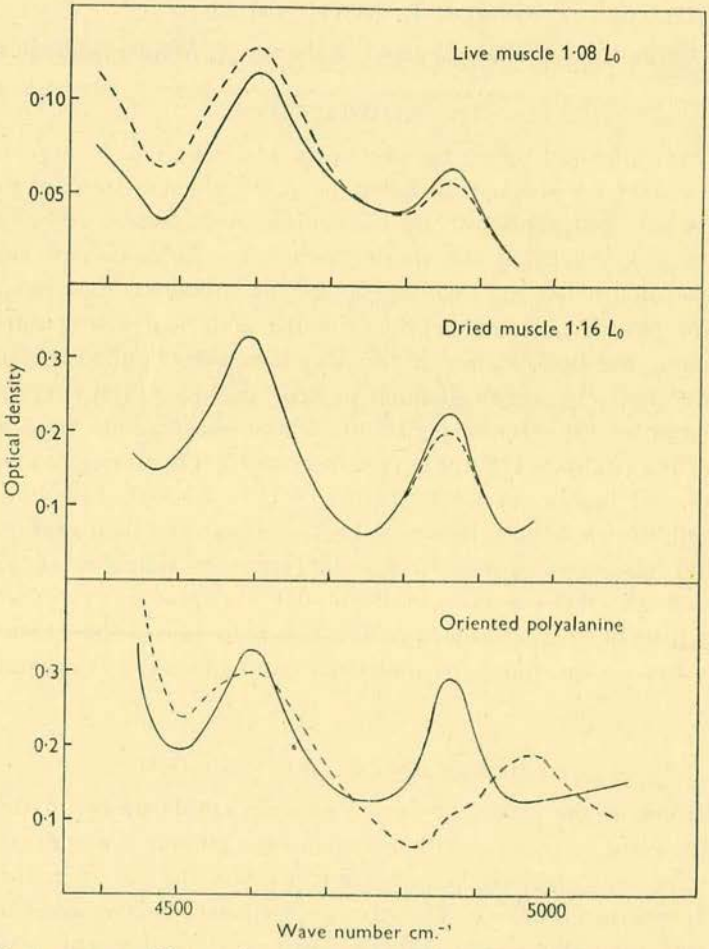

Fig. 1. Above: spectra of live resting muscle and dried muscle stretched beyond the rest length L_0 in the living animal. Below: spectrum of an oriented α polyalanine fibre. Broken line indicates measurements with the electric vector parallel to the muscle or fibre axis; full lines, measurements perpendicular to it.

(Becker & Fan, 1949). Three films of germanium prepared by evaporation on glass substrates were used, their thickness being chosen so that the variation of transmission with wave-length on account of interference gave a maximum at 4900 cm.$^{-1}$, thereby avoiding high reflexion losses. To keep the energy fairly constant throughout the spectrum and to compensate for

the edge of the strong absorption band of water at 5200 cm.$^{-1}$, the spectrometer slit was closed from 0·15 to 0·075 mm. in the range 4900–4700 cm.$^{-1}$. With these precautions it was necessary to use a selected lead sulphide cell cooled to $-78°$ C. as the detector to obtain sufficient sensitivity. Finally, it was necessary empirically to correct for the displacement of water by the protein when measuring the absorption relative to an equal thickness of Ringer's fluid. Errors in this have only a slight secondary effect on the shape and position of the protein bands and negligible influence on the dichroism. These difficulties were not encountered in making measurements on dried specimens and the results are very similar. It was nevertheless thought preferable to concentrate mainly on obtaining reliable measurements on live specimens, since results derived from dried specimens are of restricted value.

Results are also shown for a specimen dried under tension so as to produce the maximum amount of orientation. Considerable lateral shrinkage occurred on drying so that the optical density of the dried specimen is much higher than that of the live one. An X-ray photograph of the dried specimen was also obtained with a vacuum camera giving the usual type of α diagram for muscle.

These results are compared with those obtained with a polyalanine fibre with a high degree of molecular orientation, mainly in the α form but containing about 10% of extended β material. This is revealed both by X-ray photographs and by the development of a dichroic shoulder at 4520 cm.$^{-1}$ on the band at 4600 cm.$^{-1}$ in the infra-red spectrum. Since the orientation of the β material is very high and the dichroism is of the opposite character to that of folded α polypeptides, the dichroism of the NH combination band at 4860 cm.$^{-1}$ is somewhat lowered. In order therefore to compare the dichroism of muscle with that of a pure α material, the spectrum of 10% of β-polyalanine has been subtracted so that the dichroic shoulder at 4520 cm.$^{-1}$ is removed.

EXPERIMENTAL RESULTS

The spectra shown are typical of a number obtained. It will be seen that the spectra of live resting muscle and dried muscle are very similar, the main frequencies are the same and the dichroism of the NH combination band at 4860 cm.$^{-1}$ is very low but of the same character as in oriented α synthetic polypeptides. In addition to this, in live muscle there is also considerable form dichroism which disappears on drying. This causes the apparent optical density to decrease towards longer wave-lengths more rapidly when the electric vector is perpendicular to the fibres than when

The writer would like to thank Prof. A. V. Hill for his advice and instruction in dissection, and Dr A. Elliott for helpful discussions. Thanks are also due to Mr W. E. Hanby for the polyalanine and Mr L. Brown for taking the X-ray photograph.

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The mounting of fibres and organic crystals for spectroscopy in the near infra-red region

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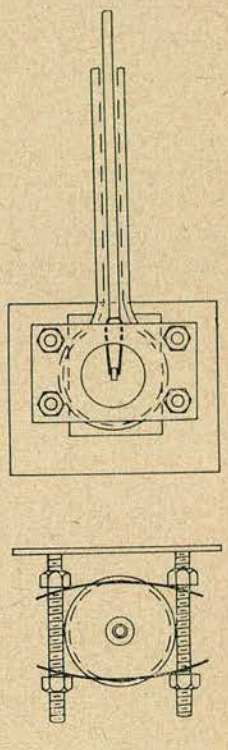
In order to obtain satisfactory absorption spectra with polarized radiation in the 1·5 μ to 3 μ region, specimens should usually be 0·1 to 1·0 mm in thickness. With fibres, a number have to be mounted parallel and in an immersion medium to reduce the amount of scattered radiation; if very fine fibres are being examined the number required is considerable. For single crystals an immersion medium is desirable and it is necessary to be able to mount the crystal in a particular orientation. The following simple techniques have been found to work satisfactorily when used with a spectrometer fitted with a reflecting microscope giving ×5 magnification.

For mounting fibres, narrow tapered tubes are first made by drawing points on 0·5 cm diameter glass tubing. These can be made in pairs with the points at the ends sealed off, and thereby kept clean until needed. Lengths about 2·5 cm long and tapering from about 3 mm to 0·5 mm are then cut, both ends being open. The ends of a loop of fine wire are pushed down from the wide end of the tube and the fibres to be examined passed through the loop. They are then pulled down tightly into the tube by the wire so that they become parallel. The tube is filled with an immersion liquid by placing a few drops in the wide end which are then drawn down by capillary attraction, air bubbles being removed by placing the specimen in a vacuum desiccator which is partially evacuated a few times. With hexachloro-1,3-butadiene which is commonly used there is no need to seal off the tube and having both ends open assists filling. It is almost inevitable that there should be some residual scatter of radiation which has to be compensated for if a "cell in—cell out" or double-beam system of recording is used. For this purpose a piece of glass ground with carborundum is useful, a final match being obtained by rubbing a black-lead pencil on the ground glass.

A spherical cell is used for single crystals, made by blowing a bulb of glass on the end of a glass tube of about 4 mm outside diameter. The bulb should be reasonably strong, as uniform and spherical as possible and about 1·3 cm to 1·5 cm in diameter. The crystal is held in the centre by a pair of narrow springy tweezers about 1 cm long, soldered on the end of a stiff brass wire about 16 s.w.g. so that it will slide comfortably down the glass stem. The tweezers are made from thin springy metal and the points should normally press together. By slightly flattening the wire in two or three places, it can be made to slide fairly stiffly down the glass tube so that the crystal is held as nearly in the centre of the sphere as possible. To pick up a crystal, the tweezers are best opened with a smooth tapered probe which thus assists also in steadying the points. The cell is filled with carbon tetrachloride or other immersion liquid and the crystal carefully inserted, the top then being sealed with fish glue. The bulb is gripped between a pair of thin beryllium/copper strips 1·3 cm wide in holes about 0·9 cm in diameter through which the radiation passes. The strips are held in turn on a rectangular brass plate with a larger rectangular hole in it as shown.



Elevation and plan of cell assembled with a crystal inside

Mounted in this way the orientation of the crystal can be adjusted on the stage of a polarizing microscope before transfer to the spectrometer. Provided that the aspect it is desired to examine is not obscured by the tweezer points, a considerable range of rotation and tilting can be obtained and a satisfactory image projected on the spectrometer slits of specimens with dimensions of the order of 0·5 mm.

By the use of a spherical cell the full magnification of the reflecting microscope is maintained and there are no serious aberrations. The cell forms a simple type of Federov mounting and can also be used for qualitative microscopy in the absence of a more elaborate universal stage.

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Absolute Configuration and Optical Rotation of Folded (α) Polypeptides

SINCE Pauling and Corey[1] proposed the α-helix for the structure of some folded polypeptides, evidence for the essential correctness of this structure has accumulated; but agreement between the observed X-ray diffraction pattern of oriented fibres and that calculated from the model has not been obtained. As Brown and Trotter[2] have recently shown, with α poly-L-alanine ($(CO.CH.CH_3.NH)_n$), where the scattering centre of the side-chain is fixed with respect to the helix, the agreement along the layer lines is very poor. In this case, the X-ray reflexions can be indexed on a unit cell through which only one chain passes, and it was assumed that both the sense of the helix (right- or left-handed) and the direction of the peptide sequence were identical for all chains. The fit was so poor that it could not be concluded with certainty whether the helices were right- or left-handed.

Since the α-helix conforms accurately to the conditions for minimum energy and also has dimensions which are almost exactly those required to fit the unit cell of poly-L-alanine, it seems likely that the atomic parameters of this helix must be very nearly correct. We have accordingly examined more general arrangements of eighteen-residue, five-turn helices (fibre repeat 27 A.), using the reflecting optical diffraction spectrometer which was described recently[3]. The fibre repeat in poly-L-alanine is longer than this[2], but the effect of the longer repeat on the optical transform is not appreciable. Like-handed hexagonal arrangements of helices which have near six-fold screw axes pack with the observed inter-chain distance whether all chains have the same direction of peptide sequence or not; the necessary condition is that the β-carbon atoms of the methyl side-chain groups should be in identical crystallographic positions. A random arrangement of the direction of the peptide sequence of the individual chains (which is very probable in the absence of strong polarizing forces) will produce the diffraction pattern of a primitive unit cell, though there is, in the strict sense, no unit cell. This arrangement of right-handed helices (corresponding to βC_1[4]) gives diffraction patterns in much better agreement with the observed X-ray pattern than does a left-handed one. The refinement of co-ordinates is not yet complete, but enough has been done to show that satisfactory agreement may be expected with quite minor changes

in the co-ordinates originally given by Pauling and Corey[5]. It is most unlikely that any appreciable proportion of left-handed helices contributes to the crystallite reflexions. It is possible, however, that left-handed helices contribute to the observed layer-line streaks; but in this case the proportion would be small. We believe, therefore, that in poly-L-alanine the helices are mainly right-handed, and presumably α-helices of other L-amino-acid polymers will also be right-handed.

The specific optical rotation of an α-helix of poly-glycine (using a very simplified model) has recently been calculated by Fitts and Kirkwood[6]; for right-handed helices this is found to be +132°, in an aqueous solution with index of refraction 1·35. Poly-L-(or poly-D)-alanine is not soluble in non-polar solvents, and since in polar solvents the chains may be random coils (at least at low polymer concentrations) a direct measurement of the specific rotation of an α-helix of poly-L-alanine cannot well be made. However, a *meso* polymer containing a small proportion of one enantiomorph is slightly soluble in chloroform. From measurements on such material, the specific rotation of poly-L-alanine is about +50°, which is considerably less than the figure given by Fitts and Kirkwood for a right-handed helix without active side-groups. Unless their estimate is grossly in error, it would appear that the contribution of the side-chain opposes the form optical rotation of the helix. This would account for the generally low value of the specific rotation of polypeptides in conditions which favour the α-helix form. Fitts and Kirkwood[7] have recently, from similar considerations of optical rotation, also concluded that α-polypeptides form right-handed helices.

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Aug. 3.

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Chain arrangement and sense of the α-helix in poly-L-alanine fibres

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[Plate 4]

The X-ray diffraction pattern of poly-L-alanine fibres has been compared with optical diffraction patterns of α-helices. With bond lengths and angles not significantly different from those found in simple compounds, good agreement is found with right-handed (but not with left-handed) helices. It is necessary to suppose that the direction of the peptide sequence of chains in the crystallites is random. Helices with a long repeat distance are found to pack in a way which produces a limited sequence of residues, spaced at 4·5 Å, in which displacements from steric effects can be expected. It is shown how this may produce a meridian reflexion at 4·4 Å, as observed. A complete account of all features of the packing is not given, however, and hardly seems to be practicable.

Introduction

Although the general correspondence between the X-ray diffraction pattern of the α-helix of Pauling, Corey & Branson (1951) and that of several folded synthetic polypeptides has been recognized for some years, a detailed and satisfactory agreement has not yet been reported. The general correspondence is in fact only evidence of the helical nature of the fold, with a helix pitch of 5·4 Å and a residue translation of *ca.* 1·50 Å, and does not establish the positions of the atoms as those of the α-helix. Indeed, the optical transform of the $3{\cdot}6_{11}$ helix (Huggins 1952; Elliott & Robertson 1955) might seem to afford a better starting-point for explaining the X-ray diffraction pattern of poly-L-alanine than do the α-helix structure amplitudes which have been given by Brown & Trotter (1956) and by Pauling, Corey, Yakel & Marsh (1955). We have already indicated briefly our belief that the wrong choice of unit cell has been responsible for lack of agreement in the case of poly-L-alanine (Elliott & Malcolm 1956).

Some years ago the preparation of well-oriented fibres of poly-L-alanine in the α-helix form was reported from this laboratory, and an X-ray diffraction photograph was reproduced (Bamford, Brown, Elliott, Hanby & Trotter 1954). A more complete examination of the diffraction pattern was carried out by Brown & Trotter (1956) who measured interplanar spacings and the relative intensities of the stronger reflexions. They showed that the unit cell is hexagonal, with side $a = 8{\cdot}55 \pm 0{\cdot}03$ Å, and that the *c*-axis (chain repeat) is long. The arguments, based on the stereochemistry and on the diffraction patterns of α-helices (Pauling & Corey 1951; Cochran, Crick & Vand 1952) show that the minimum value of *c* compatible with the measurements is 70·3 Å; this is the repeating length of an α-helix with 47 residues in 13 turns, the pitch being 5·41 Å and the axial translation $1{\cdot}495 \pm 0{\cdot}003$ Å per residue.

The number of residues per turn is $3{\cdot}615 \pm 0{\cdot}003$, corresponding to a residue rotation of 99° 34′. There are no crystal reflexions in the diffraction pattern to suggest that the unit cell has more than one helix passing through it, and Brown & Trotter considered the diffraction pattern of such a primitive cell, and calculated the intensities of many of the reflexions for the two possible screw senses (right-handed helices corresponding to Pauling & Corey's position 1 for the β carbon atom in poly-L-alanine). They showed that, with co-ordinates for the atomic positions closely similar to those given by Pauling & Corey (1951), quite good agreement between observed and calculated intensities was obtained for the equatorial reflexions, but that in general the agreement was poor elsewhere. It was considered that rather better agreement was obtained with the left-handed sense, but it was noted that better agreement over all the layer lines would be needed to substantiate this tentative assignment. Brown & Trotter discussed the origin of the strong layer-line streaks which appear on two of the layer lines (not on the equator) and suggested that they were caused by a random displacement of some of the chains in the direction of the chain axis, which as is well known would produce layer-line streaks while leaving the equatorial reflexions sharp. However, we are of the opinion that such random displacement is sterically impossible with a fixed inter-chain distance, and have proposed a different kind of random structure, based on a study of the problem of chain packing. The diffraction pattern of the proposed arrangement agrees much better if right-handed helices rather than left-handed helices are assumed for poly-L-alanine (Elliott & Malcolm 1956).

The model for the crystal of α poly-L-alanine which we shall here discuss in greater detail is a hexagonal, close-packed array of right-handed helices in which the polar sequence CO.NH.CH*R* of an individual chain may point in either direction along the helix axis, that is, the chain sense is random. This is based on the observation that, with bond angles, bond directions and van der Waals radii in common use, the packing of α-helices of polyalanine is governed by methyl group contacts. If all the helices are like-handed, and if the methyl groups have spherical shapes, any one may have its chain direction reversed without changing the packing. It is easily seen that the best packing is obtained when all the β-carbon atoms occupy identical crystallographic positions. We shall return to this matter later.

A structure such as we propose for the crystals of poly-L-alanine will evidently give sharp diffraction spots on the equator of the fibre photograph. Elsewhere, as has been shown by Cochran (1957), the diffraction pattern consists of sharp reflexions at reciprocal lattice points corresponding to a virtual unit cell containing one chain and of layer-line streaks. By using optical diffraction measurements we have found that the observed X-ray diffraction pattern (both the sharp and diffuse parts) can be accounted for by the proposed structure to an extent which justifies the adoption of right-handed helices as a structural unit in the crystal of poly-L-alanine, and the rejection of the left-handed form.

During the past two years a considerable amount of experimental and theoretical work has been done on the dispersion of the optical rotation of synthetic polypeptides in the α-helix form in solution. (Yang & Doty 1957; Moffitt & Yang 1956; Fitts & Kirkwood 1956; Moffitt 1956). This work appeared to establish the right-handed

form as the stable one in solution of poly-γ-benzyl-L-glutamate in not too polar solvents. More recently, however, the theory on which this conclusion was based has been questioned (Moffitt, Fitts & Kirkwood 1957). Optical rotation measurements do show, however, that in several synthetic polypeptides in solution one screw sense is dominant (Elliott, Hanby & Malcolm 1956; Downie, Elliott, Hanby & Malcolm 1957). This work has recently been extended to films of poly-L-alanine in the α-helix form (Elliott, Hanby & Malcolm 1957). They usefully supplement the results of X-ray diffraction, for at one time it was by no means certain that both screw senses might not occur in appreciable (if not equal) numbers in one optical isomer. According to Huggins (1952), stereochemical considerations are sufficient to warrant a choice of the right-handed as the stable form in L-polypeptides, but Donohue (1953) has doubted whether such a choice is justified.

Optical diffraction patterns

We have based our conclusions on a comparison of the X-ray diffraction pattern with the optical one produced by a mask containing holes representing the atomic positions in a chosen projection, or 'optical transform' as it is usually called (Lipson & Taylor 1951). The diffraction arrangement using a mirror of 7 in. diameter and 30 ft. radius of curvature has already been described (Elliott & Robertson 1955). Although poly-L-alanine has an α-helix of the 47/13 type, the layer lines in the X-ray diffraction pattern lie extremely close to those for an 18/5 helix, and the diffraction patterns of the two helices will be almost identical (Cochran *et al.* 1952). For reasons of convenience in drilling masks we have, therefore, used the 100° residue rotation of the 18/5 helix. In general, three repeating units of this helix were drilled to form the mask of a chain long enough to give sharp layer-lines.

In order to obtain the transform of the structure which contributes to the sharp reflexions, the transforms of the 'up' and 'down' chains are to be *added*, whereas the *difference* is taken in order to derive the transform which contributes to the layer-line streaks (Cochran 1957). The sum is obtained quite simply by putting an 'up' and a 'down' chain on a common set of axes in such a way that the βC atoms of one chain coincide with those of the other, in accordance with the model derived from considerations of packing. The projection of this fictitious chain is used to make an optical transform which when sampled at the reciprocal lattice points of a one-chain unit cell gives the squares of the structure amplitudes of the corresponding sharp X-ray reflexions, for point atoms. The three-dimensional Fourier transform of the chain has an 18-fold rotation-axis, and central sections 10° apart will, if properly chosen, show the maximum difference which any central sections have. We have examined two such sections and over the region with which we are concerned the differences are not detectable. It is therefore permissible to derive the data for all (*hkl*) values from any central section. In order to make a more quantitative comparison between optical and X-ray diffraction patterns than is possible by visual inspection, we have photographed a density scale on the plates of the optical patterns and made a photometric measurement of relative intensities at the appropriate places.

The difference of the transforms of the two chains is obtained from a mask in which the origin of one chain is given a translation d perpendicular to the chain axis. Along row lines on the optical transform where the ξ value is $1/d$, this displacement produces a phase-change 2π in the contributions from the individual chains. Hence along row lines where ξ has the values $1/2d$, $3/2d$, $5/2d$, etc. the contributions differ in phase by π, that is, along these lines we obtain the *difference* of the transforms of the individual chains.

The fictitious or virtual chain obtained by superposition on a common axis possesses a twofold rotation axis perpendicular to the chain axis through each βC atom, consequently a mask made by projection on a plane normal to such a dyad will possess a centre of symmetry. The phases of all parts of the optical transform of such a mask are therefore easily found by the well-known procedure of drilling a large hole at the centre of symmetry (Hanson, Lipson & Taylor 1953). We have in this way determined the phases, both for helices of individual atomic species and for the whole structure. This has been of considerable help in deciding what changes in the co-ordinates were likely to improve the fit of the two sets of diffraction data.

Atomic scattering factors

We have used the usual approximation for work of this kind and have adopted a unified scattering factor to correct the values of intensities derived from optical transforms for the falling-off with increasing angle. Drill sizes are then chosen to represent the average relative scattering power of the atoms over the range of scattering angle. Since it is necessary to use very small drills in order to avoid overlapping of holes in the mask, the choice of size is limited and we have used a hole diameter of 0·018 in. to represent C and CH, and 0·021 in. for NH, O and CH_3, corresponding to scattering powers 6 and 8. This gives less scattering power to the oxygen atoms than has been used in some work (e.g. Daubeny, Bunn & Brown 1954), but reference to McWeeny's curves (1951) shows the factors to be reasonable for reflexions from planes which make a large angle with the helix axis, because of the asymmetry in the scattering of the atoms of the C=O group. The reflexions from some of these planes are rather sensitive to the value assumed for the atomic scattering factor because a good deal of cancellation of contributions occurs. This happens to a much less extent with the equatorial reflexions.

Screw sense of the α-helix

Polypeptide chains made from a single optical isomer of an amino acid may be coiled into α-helices with either a right- or a left-handed screw sense, but these helices are not mirror images of each other, and the optical transforms of these two structures are appreciably though not strikingly different. If a single screw sense is present, but the chain sense is random in a crystallite, then as explained above the intensities of crystal reflexions can be derived from the transform of a fictitious structure in which the helices of opposite chain sense are superposed on a common axis. The transform of such a structure of right-handed helices is very different from that of left-handed ones. When the b-axis projection of the left-handed form

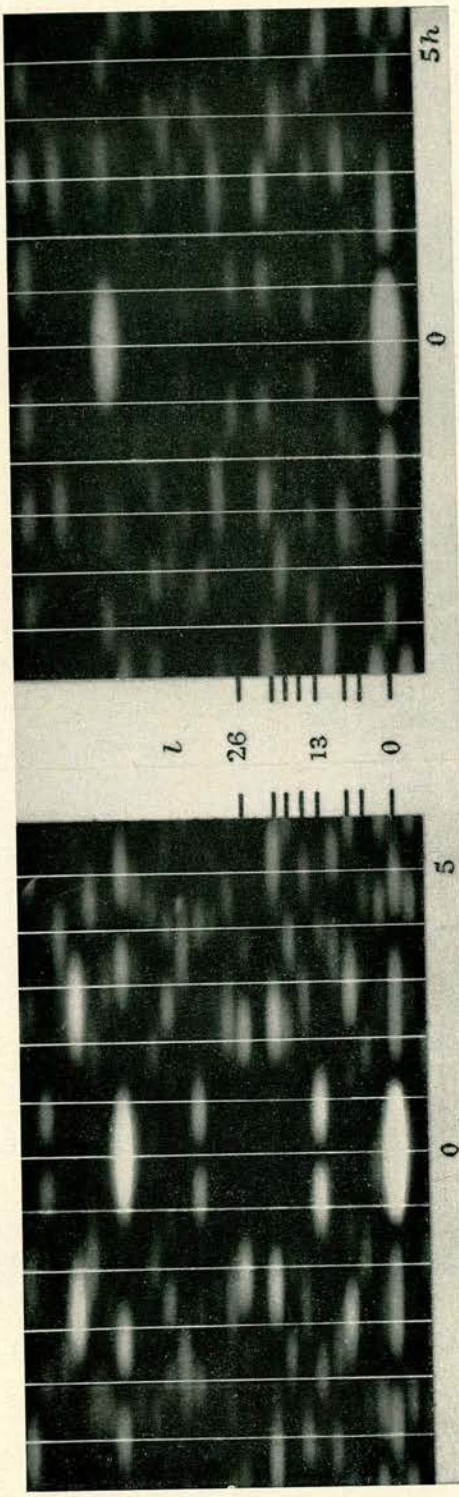

FIGURE 1. Optical transform of parallel and antiparallel α-helices of poly-L-alanine superposed on common helix axis with β-carbon atoms in identical positions. *a*, left-handed helices; *b*, right-handed helices.

(*Facing p.* 34)

of superposed helices of poly-L-alanine is compared with that of the right-handed form, the former is seen to have well-marked alternations of high and low density at intervals of the helix pitch, whereas the density of the right-handed form is much more uniform. In fact, with right-handed helices much of the diffraction pattern is contributed by the CH_3 groups (two on each site, because of the two helices of opposite chain sense), and the contributions of the other atoms cancel out over a considerable part of the diffraction pattern except on layer planes $l = 0$ and $l = 47$ (this last corresponding to the 1·5 Å axial translation).

The optical transforms of superposed antiparallel helices (corresponding to a random chain sense in the crystallite) are shown in figure 1, plate 4, for right- and left-handed screw senses, respectively, with white lines to indicate the $h0$ row lines. The turn layer line ($l = 13$), for which the calculated structure amplitude was much too high in Brown & Trotter's one-chain unit cell, is now reduced to about the right intensity in our optical transforms for right-handed but not for left-handed helices. From this it follows that with right-handed helices the contributions to this layer line from the helices with opposite chain senses are nearly out of phase. In consequence, the *difference* of the transforms will be quite large and accordingly a strong streak is to be expected. It is on this layer line that a very strong streak is observed in the X-ray photograph. With the assumption of left-handed chains, on the other hand, it is the crystal reflexions which are strong, and the streak would be very weak. These considerations, and a detailed comparison in other parts of the transform, leave no doubt that the right-handed helix is the one which occurs in poly-L-alanine.

There are some differences in the equatorial reflexions in figures 1 *a* and *b*. These are in part caused by the difference in the radial co-ordinate of the β-carbon atom in the two forms (Brown & Trotter 1956; Pauling *et al.* 1955) and also by some small changes which we have introduced in the co-ordinates to improve the fit (see below).

Meridian reflexions

The most striking evidence of the presence of α-helices in synthetic polypeptides has been the observation of the 1·5 Å meridian reflexion (Perutz 1951). This reflexion and its orders should be the only ones on the meridian in the diffraction pattern of the α-helix. However, meridian reflexions at 4·33 Å (poly-γ-methyl-L-glutamate) and 4·4 Å (poly-L-alanine) have been observed, and have been ascribed to distortions of the α-helix (Brown & Trotter 1956). Meridian reflexions from a well-oriented fibre tilted for the Bragg angle are very much stronger than reflexions from other planes for a given value of structure amplitude, and the distortion needed to produce the weak meridian reflexions which have been observed are probably small. It is easily seen that the pattern of contacts in an 18/5 helix has sixfold screw symmetry (see section on the packing of α-helices) and if some distortion in the z-direction occurred at these contacts, the electron density would have a periodic variation along the z-axis and a reflexion at 4·5 Å might be expected. However, the observed reflexion occurs at 4·4 Å. This can be explained in terms of a 47/13 helix, for as shown later there is a limited succession of places in each repeating unit, spaced at intervals of 4·5 Å along the z-axis, where appreciable distortion occurs because of packing difficulties. Although the periodicity of this variation is 4·5 Å, an appreciable X-ray

reflexion will only appear at reciprocal lattice points of the crystal; there is no lattice point corresponding to $1/4{\cdot}5\,\text{Å}^{-1}$, the nearest being (00.16), corresponding to $1/4{\cdot}4\,\text{Å}^{-1}$. If the succession of regions of distortion 4·5 Å apart were to extend without a break throughout the whole of a large crystallite, the transform of the distorted helix would be restricted to a region on the c^*-axis very near to $1/4{\cdot}5\,\text{Å}^{-1}$, and no (00.16) reflexion would be seen. Because, however, there are only about twelve successive regions of distortion in one repeating unit, the transform is finite for some distance along the c^*-axis on either side of $1/4{\cdot}5\,\text{Å}^{-1}$. The situation may be seen from figure 4, where in (*a*) the values of $|F|^2$ are plotted against ζ for a linear grating of 12 elements spaced at 4·5 Å. As may be seen, the maximum at $1/4{\cdot}5\,\text{Å}^{-1}$ is broad, because of the small number of grating elements. Figure 4 shows in (*b*) how the interference function of a crystallite containing an arbitrarily chosen number of helix repeat units (six) varies along the c^*-axis. This function has principal peaks corresponding to the reciprocal lattice points, two of which are shown. Even with less than six helix repeating units in the crystallite, it is clear that no reflexion would appear at $1/4{\cdot}5\,\text{Å}^{-1}$, since the intensity of reflexion depends on the products of the two functions shown in figure 4, and for curve (*b*) the ordinate is near zero at this point. A reflexion at $1/4{\cdot}4\,\text{Å}^{-1}$ should appear, however, and this is observed. It might appear from the figure that a (00.15) reflexion would be seen; this has not been observed. It would be less than half as strong as (00.16), and in any case it must be remembered that the details of figure 4 cannot be known accurately. We think that this explanation of the 4·4 Å reflexion is correct in principle, though to work it out in detail is hardly possible.

The packing of α-helices

It is convenient to consider first the hexagonal packing of 18/5 helices in which the methyl groups are represented as hemispheres with a radius of 2 Å. Inspection of models shows clearly that the packing is closest when all the helices are similarly oriented with the radius vector of the β-carbon atoms along the hexagonal directions. This arrangement is shown in plan in figure 2, where the β-carbon atoms on each helix have been numbered consecutively along the polypeptide chain, the difference between two numbers then representing the separation in the z-direction in units of 1·5 Å. The nearest neighbours of the three βC atoms, numbers 1, 12 and 5 on the helix at the left-hand side, have been connected by lines. Number 1 has two neighbours 3 Å above and below it as shown by full lines, numbers 12 and 5 both have one 1·5 Å and one 3 Å away in the z-direction shown by broken lines. This pattern of contacts then repeats at another level with six-fold screw symmetry as we proceed round the circle. With an interchain distance of 8·55 Å and a radial co-ordinate for βC of 3·17 Å the distance between the atoms 1, 12 and 5 and their neighbours indicated is 4·0 Å in all cases; the surfaces of the methyl groups are therefore just in contact.

This structure may be modified to take into account helices with a longer repeat or even totally irrational helices. Each atom has now to be rotated around the axis of the helix to which it belongs by an amount proportional to its number. It might be supposed that serious steric interference would arise when atoms differing in number

by unity eventually lie opposite each other in the plan along one of the hexagonal directions, but this situation does not arise since it would require a different amount of rotation for adjacent helices. The closest approach that is made between atoms differing in number by unity is 4 Å. From figure 3 it is obvious that the closest contacts which can arise are between the nth methyl groups on one helix and the

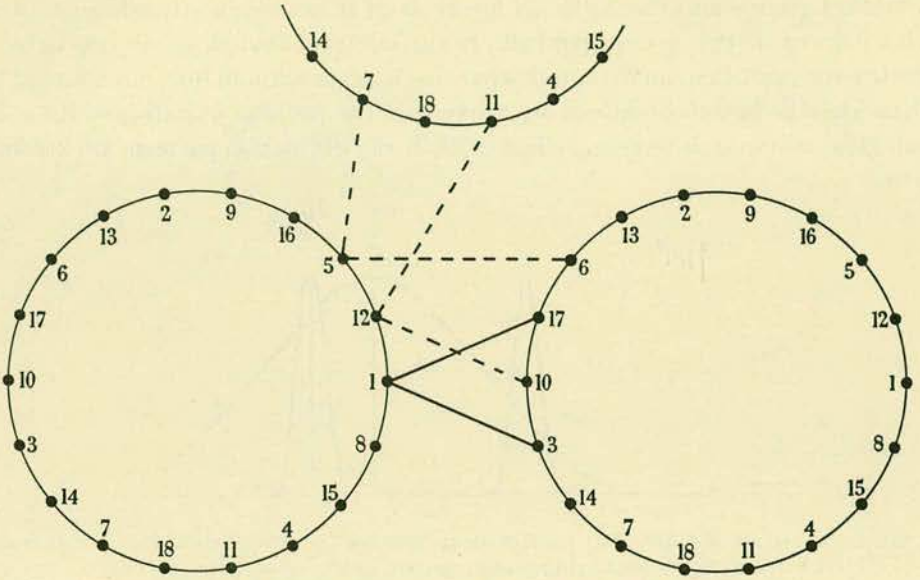

FIGURE 2. To illustrate hexagonal packing of 18/5 α-helices. Numbers indicate successive β-carbon atoms.

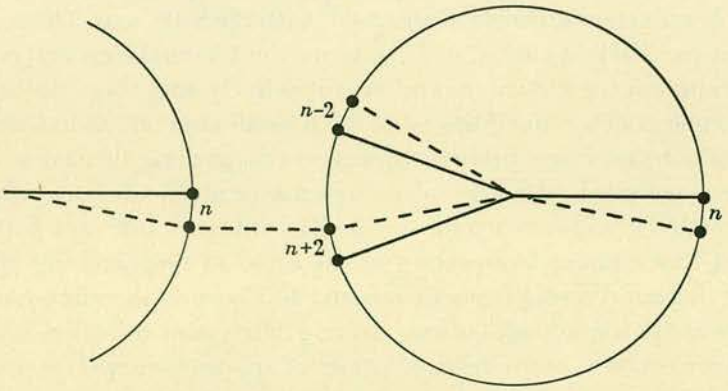

FIGURE 3. Packing of helices which do not repeat exactly after eighteen residues.

$(n+2)$th on a neighbouring one. The relationship of these atoms to each other is nearly the same as in the 18/5 helix, differing by about 1° in the case of the 47/13 helix. All cases of contacts can be examined by supposing the full lines to rotate into a new position, and it is evident that the maximum steric hindrance will occur when the line joining n with $(n+2)$ is along the hexagonal direction, as shown by the broken lines. This corresponds to a distance between methyl groups of 3·77 Å. This is shorter than the 4·0 Å distance usually found, and may be expected to give rise to appreciable distortion of the structure.

Still considering a close-packed arrangement of hemispherical methyl groups, we suppose these are on identical helices which repeat after about 70·3 Å and which now have no hexagonal symmetry elements. If the radius vector of the 1st β-carbon atom is directed along one side of the cell, the packing will be without appreciable strain at first. Examination shows that appreciable hindrance occurs between the 7th and 9th methyl groups and thereafter at intervals of three residue-translations, some twelve pairs of methyl groups per helix being involved, though to varying extents. After the 40th and 42nd methyl groups packing is again without hindrance and at the 48th residue the initial conditions are arrived at. The peculiar variation of the steric strain gives rise to an interesting effect in the X-ray diffraction pattern, as explained above.

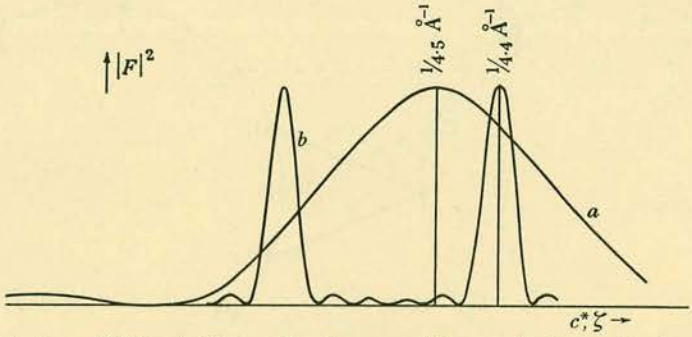

FIGURE 4. Variation of diffracted intensity along meridian. *a*, twelve scattering points spaced 4·5 Å apart; *b*, six scattering points spaced 70·3 Å apart (*c*-axis repeat).

The van der Waals surface of a methyl group is not expected to be spherical, and the αC—βC bond makes an angle of about 60° with the helix axis. The consequence of this is that parallel and antiparallel chains may not have their β-carbon atoms at identical z-values in the virtual unit cell and it is likely that they will be displaced relatively to one another along the z-axis by a small amount, as indicated by the X-ray diffraction data (see below). However, the packing of chains with their polypeptide sequences in the same sense does not produce any such displacement even if the methyl groups are not spherical; all such helices still pack with identical z-values and this remains irrespective of the sense of neighbouring chains. The sequence of displaced methyl groups referred to above is therefore not modified where chains are in contact with others running in the same direction, but it will be modified where contacts occur between chains of opposite sense. This may increase or decrease the strain at a particular contact and introduces a random element into the criteria for determining the number of strained βC atoms in a sequence. However, qualitatively the earlier model still holds, but the strain may not now build up so uniformly as before. The lack of a detailed knowledge of the shape and size of the methyl groups makes it impracticable to examine this further and also to predict the displacement of opposed chains along the z-axis. The predicted displacement varies in magnitude and even in direction with the van der Waals radius of the individual hydrogen atoms, for the rotation of the methyl group is free if this radius is 1 Å, but not free with somewhat larger radii (which, however, are within the range of hydrogen van der Waals radii recorded; see, for example, Stuart (1952)).

Discussion

The values of $|F|^2$ for the strongest layer lines up to the 26th have been derived by photometry of the continuous optical transform, and are shown plotted against the reciprocal co-ordinate ξ in figure 5. The ordinates include the square of the atomic scattering factor for carbon and a scaling factor to fit them to the observed values of $|F|^2$ for the equatorials. The co-ordinates of the α-helix are given in table 1 and figure 5 is based on these. No temperature factor has been used.

Table 1. Co-ordinates of superposed α-helices in unit cell of poly-L-alanine

$a = b = 8{\cdot}55$ Å, $c = 70{\cdot}3$ Å; helix axis along c.

	helix I			helix II		
	r (Å)	ϕ (deg.)	z (Å)	r (Å)	ϕ (deg.)	z (Å)
αC	2·29	20·7	−0·81	2·29	−20·7	0·41
N	1·49	49·7	0·06	1·49	−49·7	−0·46
C′	1·63	94·9	−0·40	1·63	−94·9	0
O	1·98	104	−1·58	1·98	−104	1·18
βC	3·17	0	0	3·17	0	−0·4
C*	2·29	120·27	0·69	2·29	−120·27	−1·09
helix axis	0	—	—	0	—	—
b axis	—	0	0			

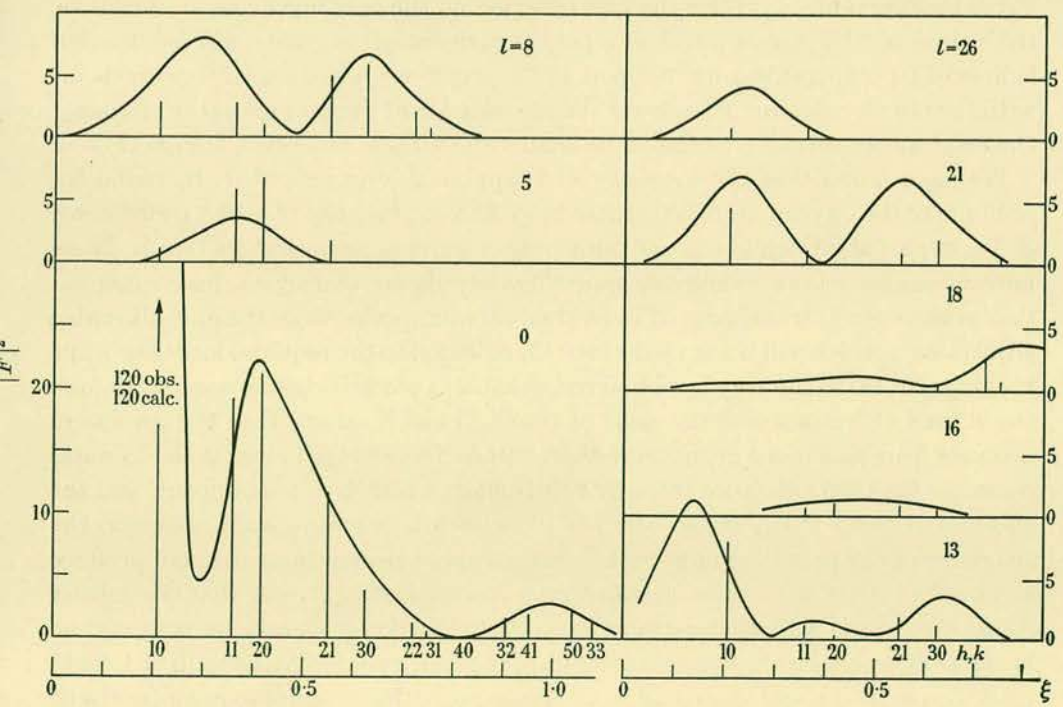

Figure 5. Values of $|F|^2$ for crystal reflexions on chief layer lines in diffraction pattern of α poly-L-alanine. Full line gives values from photometry of optical transform. Observed values for reciprocal lattice points from Brown & Trotter (1956).

If the atomic co-ordinates given by Brown & Trotter or the very similar ones of Pauling *et al.* (1955) are used to derive $|F|^2$, and the β-carbon atoms in all chains (parallel and antiparallel) are given identical co-ordinates, the fit with the observed values of $|F|^2$ on the 13th layer line is poor. We have tried many small adjustments to rectify this; the only effective one is a change of about $+0{\cdot}2$ Å in the z-co-ordinate of the O, C′, αC and N atoms of chain I, with a corresponding change for chain II in the opposite direction. This might mean that the z-co-ordinate of the β-carbon atom in each chain is altered with respect to the other atoms in the peptide residue, while the β-carbon atoms of chains I and II occupy identical positions in the virtual unit cell. This, however, leads to bond angles and lengths considerably different from those usually found in other compounds. A more likely interpretation is that the oppositely directed chains are displaced by about 0·20 Å in opposite directions, for reasons already discussed. Although the two possibilities should be distinguishable, since the β-carbon atoms are not in the same place in each case, the small differences in the optical transform which are seen are insufficient to allow a choice to be made.

The fit for $l = 0$ is good. The (11.0) reflexion is too strong, but this is accounted for by the fact that it is overlaid by a reflexion from the β phase, present in a small amount. Elsewhere the agreement is moderate, and perhaps as good as can be expected, having regard to the fact that the intensity on most of the layer lines is weakened by interference from the contributions of the two chains. This is seen by comparing with the much higher $|F|^2$ values for single chains (Brown & Trotter 1956; Pauling *et al.* 1955); for the first reflexion on the turn layer line, for instance, the values of $|F|^2$ for βC_1 and βC_2 (which correspond to right- and left-handed helices of L-polypeptides) are 23·2 and 46·7 respectively when scaled to correspond with the (10.0) reflexion. Because of the cancellation of a great part of the intensity, the resultant is sensitive to the co-ordinates and atomic scattering factors chosen.

We have found that the intensity fit is appreciably improved if the radial co-ordinate of the oxygen atom is increased to 1·98Å (an increase of 0·20 Å over Brown & Trotter's value), while that of the nitrogen atom is decreased to 1·49 Å. These movements leave the amide group approximately planar, and appear from examination of the optical transforms of individual atomic species to be the only allowable adjustments which will bring up the (30.13) reflexion to the required intensity while keeping (20.13) to the very low observed value. If a plane is chosen passing through the αC and αC* atoms and the mean of the C′, O and N atoms, then the maximum distance from this plane is not more than 0·04 Å. The change in radial co-ordinates increases the C=O distance from 1·24 to 1·265 Å, which is not significant, and the effect of moving the nitrogen atom is likewise without significant effect on the geometry of the peptide group. Small changes in the ϕ co-ordinates do not produce any marked effect on the optical transform. It is interesting to note that the α-helix shows some steric hindrance between the carbonyl carbon atom and the oxygen atom in the next residue (O*) when these atoms are given van der Waals radii of 1·4 and 1·8 Å, respectively, and this could be the reason why the oxygen atom appears to be at a greater radius than expected, in order to relieve the packing. We are obliged to Dr Conmar Robinson for having drawn our attention to this.

Considerable support to the scheme we propose for poly-L-alanine is given, we think, by the prediction of the layer-line streaks from the difference of the transforms of the parallel and antiparallel chains (figure 6). On the turn layer line ($l = 13$) the maximum of the observed streak is just where the continuous curve has its maximum, at the (10.13) reflexion, and the weaker streak on $l = 21$ is observed at

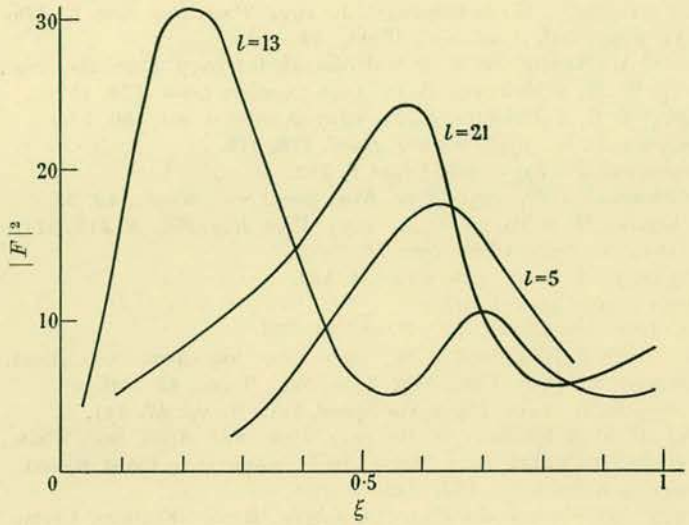

FIGURE 6. Values of $|F|^2$ for layer-line streaks, from photometry of optical transform.

considerably greater values of ξ, as expected. The only other streak which conceivably might be seen is on $l = 5$; this is predicted to be weak, and being near the equator and rather far out there will be geometrical factors affecting its intensity compared with the other streaks: it has not been recorded.

CONCLUSION

The correspondence between the values of $|F|^2$ derived from optical and from X-ray diffraction patterns establishes the screw sense of the helix and the validity of the assumptions we have made about the random arrangement of parallel and antiparallel helices; it also provides the most complete experimental evidence, to date, for the α-helix. To examine in detail the bond lengths and angles within the peptide group would require more reliable X-ray data than can usually be obtained from fibres. It seems likely, from the way in which the optical transform depends on the atomic scattering factors chosen, that account would have to be taken of the positions of the hydrogen atoms, and this would be difficult in the case of the CH_3 groups because of the uncertainty about rotation around the αC—βC axis. For these reasons, we have not thought it useful to go from the predictions of the optical transform to calculation of the structure amplitudes.

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A sensitive photoelectric polarimeter

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[*Paper received* 29 *August*, 1956]

The construction of a simple photoelectric polarimeter is described. The instrument has a sensitivity considerably higher than the usual visual type but with a more restricted range. Features of the instrument which make for convenience in operation and also to which attention must be paid in order to use fully the intrinsic sensitivity of the system are outlined.

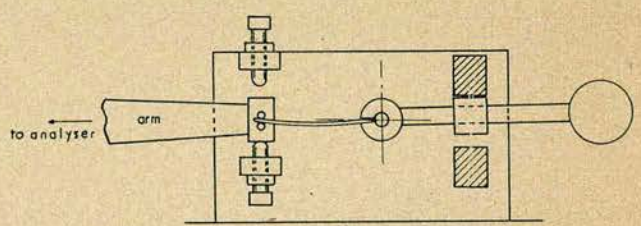
The conventional type of visual polarimeter used in most laboratories suffers from a number of disadvantages. For accurate work, the observer needs to be dark-adapted and consequently a dark-room is necessary. In addition, the sensitivity is not particularly high and often necessitates the use of a long specimen tube. The volume of liquid required is then considerable, and if the solution is at all coloured or turbid the sensitivity of the instrument is reduced. These considerations make observations tedious and sometimes difficult and may well limit the application of polarimetric methods in certain fields. It was therefore thought worthwhile to develop a simple and accurate polarimeter using a photomultiplier tube as the detector which might overcome some of these difficulties. There have been a number of descriptions of various types of photoelectric polarimeters[1–5] in recent years, of varying degrees of complexity. Many follow the photomultiplier with one or more stages of electronic amplification: this appeared unnecessary and only an attachment for a normal commercial instrument described by Rudolph[5] attains the simplicity and sensitivity of the present instrument. By building the entire instrument rather than adapting existing commercial models, the full advantages of the higher sensitivity and accuracy obtainable with a photomultiplier detector can more readily be attained.

DESCRIPTION

The general arrangement of the instrument is as follows. A lamp illuminates a pinhole which is at the focus of an achromatic lens. This provides an (approximately) parallel beam of light which passes through the polarizer, the cell and the analyser and falls on the photomultiplier. A shutter is provided to protect the photocell, and a mirror which can be lowered at 45° to the beam to look along the beam by eye. This is very useful for examining the solution and detecting birefringence in the cell windows, in stray particles or arising from the nature of the solution. An approximate extinction can also be found very quickly by eye.

The polarizer and analyser are both polarizing filters as supplied by Cooke, Troughton and Simms Ltd. in strain-free mountings for use in polarizing microscopes. These are mounted in the centres of ball-races, taking care to use a method of holding them in place which does not introduce any strain in the system. The polarizer is rotated by an arm with a ball-bearing in the end, which is loaded by an extension spring against the anvil of a 25 mm/micrometer head. It is arranged so that the line joining the centre of rotation of the polarizer bearing to the end of the ball is at right angles to the micrometer axis when the micrometer is set in the middle of its scale. If the length of this arm is 18·85 cm the micrometer reading in centimetres multiplied by three gives the rotation in degrees, and rotations of $\pm 3 \cdot 6°$ can be measured. Should the rotation be higher than this a shorter cell can be used.

The analyser is mounted so that it can be rotated through a pre-set angle by an arm as shown in the figure. The rectangular steel block on the end of the arm carries two pairs of short steel rods between which two strips of phosphor bronze $\frac{1}{32} \times \frac{1}{8}$ in. are just free to slide. These are rotated by moving the handle on the right up or down so that they press the steel block on the pre-set screws. To obtain a quick changeover a piece of soft iron can be mounted on the arm connected to the handle so that it moves between the poles of a permanent magnet (shown shaded).



Mechanical arrangement for the rotation of the arm connected to the analyser

All the components are mounted on a cast-iron base for mechanical rigidity and enclosed in a light-tight case. Apertures are provided in the top for viewing the mirror and placing the cell in position. For use by non-technical personnel it is desirable to have an interlock device to prevent the instrument being opened unless the shutter is closed.

The photomultiplier is an E.M.I. type 6094 B tube run either from a stabilized power supply or batteries and the output is observed on a Cambridge spot galvanometer with a sensitivity of 170 mm/μA. Provision is made for backing off the galvanometer. Other types of phototube may be satisfactory but the method of construction of those made by E.M.I. Electronics Ltd. should make the sensitivity independent of the direction of polarization of the incident radiation. It is very desirable that the photo-cathode should be as uniform in sensitivity as possible, since errors will occur on this account if the polarizers are non-uniform.

The supply for the lamp should also be stabilized. The authors have used a 45 W sodium discharge lamp in the "end-on" position to obtain increased brightness. This illuminates a pinhole of adjustable diameter; about $\frac{1}{16}$ in. is convenient for most work. A certain amount of light may come out of the end of the glass of the polarimeter tube itself, which will give a large standing signal. To eliminate this a stop slightly smaller than the diamater of the tube should be fitted on the end nearest to the photomultiplier.

In operation, the micrometer head is adjusted so that the

signals in either position of the arm are equal. If θ is the angle between the direction of polarization of analyser and polarizer the transmission I is proportional to $\cos^2\theta$ plus a constant small amount depending on the quality and optical density of the polarizing filters. Oscillation of the analyser gives two signals which are balanced. The rate of variation of I with $\cos^2\theta$ is a maximum when θ is $\pm 45°$; this, however, is not the optimum angle for the rotation of the analyser since there is then a very large signal on the photocell and the accuracy is limited by the stability of the source. For clear solutions, an angle of $\pm 1°$ or $2°$ is sufficient. For narrow bore micro-tubes or coloured solutions where the amount of light transmitted is reduced, this angle can be increased. The point has been fully discussed by Rudoph.[5] We have found that it is possible to obtain readings to $\pm 0{\cdot}001°$ with ease. The absolute accuracy is not as good as this and depends on the quality of the optical components and the uniformity of the photomultiplier surface. We have found, however, that the values for the optical rotation of solutions calculated from the length of the arm on the polarizer and the micrometer reading, agree well with values for the solution on a visual instrument. For coloured solutions, accurate measurements can be made where any reading is impossible on a visual instrument.

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Infrared Absorption Spectrum of Water adsorbed on α-Helical Synthetic Polypeptides

I REPORT here some observations on the polarized infrared absorption spectrum of water adsorbed on orientated synthetic polypeptide films. The results suggest that the water molecules are adsorbed at specific sites and orientations with respect to the substrate molecules.

Orientated films of high molecular weight synthetic polypeptides in the α-helical conformation were prepared by collapsing to one end of a Langmuir trough monolayers spread on the water surface. The method used to spread the monolayers and to remove the collapsed film has been described previously[1,2]. Sufficient monolayers were spread to give a specimen with an absorbance of 1·5 or greater for the amide I band. Films were mounted on barium fluoride plates and dried to a predetermined humidity. Where necessary, scatter of radiation from the surface of the specimen was reduced by allowing a drop of chloroform or benzene to flow across the surface so that it softened and became compacted. This treatment improved the quality of the spectra and probably modified the crystallinity of the specimen, but was not essential to see the main features described. Because the OH-stretching band lies close to the amide A (NH-stretching) band, it is helpful (but not essential) to reduce the NH absorption by using N-deuterated polymer and spreading the monolayers on 0·01 M HCl on which the back exchange of deuterium is slow[1].

High molecular weight specimens of poly-D-alanine, poly-γ-ethyl-L-glutamate and poly-γ-methyl-L-glutamate have been examined with particular reference to the 2,000–4,000 cm^{-1} spectral range. Although the strength and shape of the water absorption band varies from one polymer to another, three main features are common to all the polymers: (1) the OH absorption is not centred about 3,400 cm^{-1} as in liquid water, but is displaced to around 3,500 cm^{-1}; (2) the band is clearly made of two or three components; (3) overall, the band exhibits marked perpendicular dichroism. Fig. 1 illustrates these features for poly-γ-methyl-L-glutamate, together with the NH and N^2H stretching bands (about 3,300 and 2,450 cm^{-1}), which

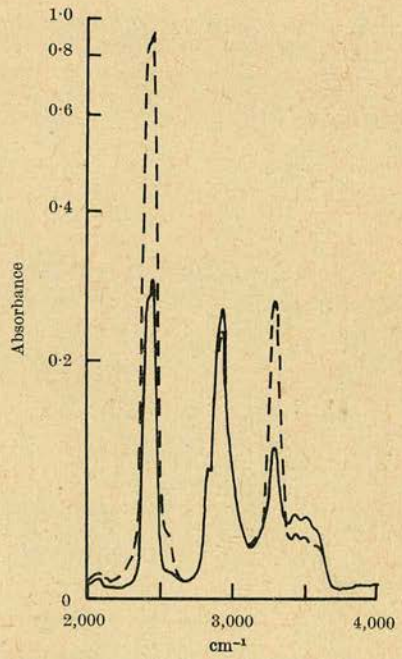

Fig. 1. Infrared absorption spectrum of a partially N-deuterated film of poly-γ-methyl-L-glutamate, 30° C, approximately 91 per cent relative humidity. Full line: electric vector perpendicular to direction of orientation; broken line: electric vector parallel.

are of opposite dichroic character to the water band, and the CH bands. The water band has components at about 3,440, 3,510 cm^{-1} and 3,570 cm^{-1}. Poly-γ-ethyl-L-glutamate has three similar bands and poly-D-alanine has a band at about 3,470 cm^{-1} with a shoulder at 3,530 cm^{-1}.

Because the frequency of the water absorption band is roughly mid-way between that of the free and hydrogen-bonded OH stretching frequencies it is probable that the hydrogen bonding component of the binding is weak. The dichroism and splitting of the band into two or three components is good evidence that the molecules are in specific orientations and sites with respect to the polymer. If the band is attributed mainly to the OH antisymmetrical stretching vibration (normally the strongest), which has a transition moment parallel to the line drawn through the hydrogen atoms, the molecules are orientated on average with this direction fairly close to planes drawn perpendicular to the helix axis. An estimate of the angle can be made by using the direction of the NH and N^2H transition moments to give a measure of the orientation

of the polymer. The usual model for fibre orientation is assumed with a fraction of perfectly orientated polymer and the rest disorientated[3], and the water is taken to be distributed in proportion over both fractions. This leads to the H–H direction being within about 25° to the plane perpendicular to the axis of the helix.

Weak perpendicular dichroism of the same water band has been observed in hydrated α-keratin by Bendit[4], who found the frequency to be 30–40 cm^{-1} higher than in liquid water. Although the spectrum was very similar to that of an equivalent thickness of liquid water, it is probable that the observations are closely related to the present work.

A tentative explanation of these observations, consistent with the views of Bendit for keratin, is that the water molecules orientate with a hydrogen atom directed towards the peptide oxygen, possibly forming a weak hydrogen bond to it, with the H–H direction of the water molecule within 25° to the plane perpendicular to the helix axis. In this situation it is probable that the water dipole will interact strongly with the peptide dipole. This orientation would account for the positive sign of the surface potential when a monolayer of α-helices is spread on water[1], if on a clean water surface the hydrogen atoms are normally directed downwards[5]. In the case of the glutamate polymers an additional interaction may occur between the water and the side chain carbonyl.

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Optical Rotation of the α-Helix in Synthetic Polypeptides

In a communication from this laboratory[1], the belief has been put forward that the polypeptide chains in poly-L-alanine fibres form right-handed rather than left-handed helices; this view is based on the X-ray diffraction pattern. If it is correct, it is to be expected that the same sense of helix will be the stable form in other L-polypeptides. Now the contribution to the optical rotation of an α-polypeptide from the helix alone is considerable; according to Fitts and Kirkwood[2], a right-handed helix of polyglycine should have a specific rotation of about + 130°. In a later communication[3] they have concluded from the change in specific rotation on destruction of the α-helix (in poly-γ-benzyl-L-glutamate and in poly-L-glutamic acid) that in these polymers the helices are right-handed.

The stability and sense of the α-helix can be examined by measuring the optical rotation of a series of polypeptides in which the proportion of D- to L-residues is varied. The addition of a proportion of D-residues, randomly arranged along a chain of L-residues in the α-helix form, will not affect the helix if this has a strongly preferred sense. The optical rotation of a predominantly L-polypeptide should therefore at first move towards higher positive values as the proportion of D-residues is increased, but must ultimately become zero when D- and L- residues are present in equal amounts.

We have measured the optical rotation of a series of D : L-leucine polypeptides in benzene at a concentration of 0·2 per cent (w/v). Films cast from these solutions showed the carbonyl infra-red absorption band in the normal position for the α-form. Since many of these polymers form gels under these conditions (even at 60° C.), small amounts of *m*-cresol (minimum 1 per cent, v/v) were added, and the optical rotation in pure benzene was obtained by extrapolation to zero concentration of *m*-cresol. This procedure was justified by measurements made in pure benzene, when the solubility allowed this. The addition of *m*-cresol lowers the positive rotation of the mainly L-polypeptides. Even with *m*-cresol present, it was found necessary to make measurements at 60° C. to avoid gel formation. It is con-

venient to express the results in terms of the residue rotation $[R]$

$$[R] = \frac{R}{100}[\alpha]$$

where R is the residue weight and $[\alpha]$ the specific rotation defined in the usual way. The results are shown in Fig. 1.

The expected effect is strikingly apparent, and the increase in rotatory power continues to a lower excess of L over D values than might have been anticipated. The increase in rotatory power is not caused by increasing instability of the α-helix as the proportion of D-residues becomes greater (leading to the formation of a random coil), because conditions which favour a random coil lower the positive rotation[4]. The linear part of the graph is readily accounted for

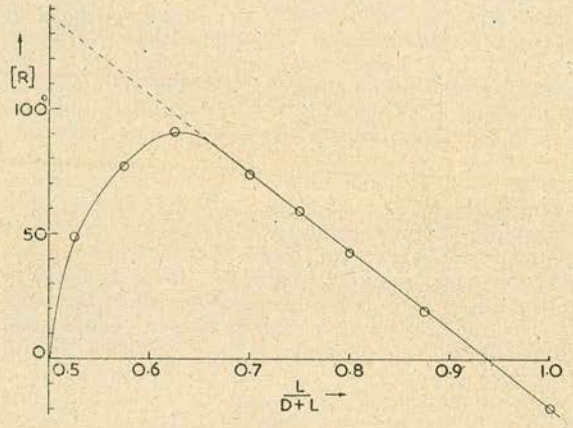

Fig. 1. Residue rotation of poly-leucine plotted against fraction of L-residues in polymer. Concentration 0·2 per cent w/v in benzene at 60° C.; $\lambda = 5893$ A.

if, over this range, all the polypeptide chains are right-handed, producing a positive rotation from the helix core, with the L- and D-side-chains giving respectively negative and positive contributions. The contribution from the helix core is given by the intercept on the y-axis, and corresponds to a residue rotation of + 138°. We may note that the corresponding value for poly-L-leucine in benzene calculated from Fitts and Kirkwood's formula is 70°.

Our experiments show clearly that, in solution, one sense of helix is significantly more stable than the other for a given enantiomorph. Together with Fitts and Kirkwood's calculation that the form

optical rotation is positive for a right-handed helix, the results confirm that L-polypeptides form right-handed helices. The possibility that the optical rotation of an α-polypeptide can be treated as the sum of contributions from the helix core and the asymmetric centres is interesting, and may have applications in the case of proteins such as silks.

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The optical rotation and molecular configuration of synthetic polypeptides in dilute solution

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The optical rotations of a number of synthetic polypeptides have been measured in a variety of solvents. For each polypeptide species, a number of copolymers of different D:L compositions have been examined. Several distinct types of behaviour have been found, which may readily be interpreted in terms of the relative stability of left-handed helices, right-handed helices, and random coils. In solvents that are not too polar (even for quite considerable departures from the enantiomorph) predominantly L polymers exist entirely as right-handed helices.

The rotation of a right-handed helical form of several *meso* polypeptides has been deduced; this rotation varies somewhat from polymer to polymer but is not markedly dependent on the solvent. It appears that the solvated D and L residues on a *meso* helical polypeptide largely cancel out each other's contributions to the rotation.

The dispersion of the right-handed helical form of poly-DL-leucine has been derived.

Introduction

The possibility that the optical rotation of a synthetic polypeptide might be dependent on its configuration was first examined by Robinson & Bott (1951), who showed that the optical rotation of a 1:1 copolymer of γ-methyl-L-glutamate and DL-phenylalanine is strongly dependent on the solvent used. They found in a series of polymers that the positive optical rotation increased with the molecular weight when the solvent was *m*-cresol, but that a nearly constant negative rotation was observed in formic acid. Films cast from these solvents were examined in an infra-red spectrometer, and it was found that, whereas all the films cast from formic acid were in the β (extended chain) form, those from *m*-cresol contained an amount of α (folded) polypeptide which increased with molecular weight, becoming 100% at the highest molecular weight examined. They suggested that the optical rotation gave an indication of the fraction of α material in the solution.

Since this pioneer work, much more has been discovered about the α configuration of simple polypeptides, in which the chains are helical, and which must therefore be optically active if all the helices are like-handed. In non-polar solvents it is to be expected that polypeptides of high molecular weight will form the α helix of Pauling, Corey & Branson (1951), and observations of infra-red spectra of such solutions support this in the case of poly-L- and poly-DL-leucine (Elliott 1953) and poly-γ-benzyl-L-glutamate (Doty, Holtzer, Bradbury & Blout 1954). For the latter polypeptide, additional and convincing evidence is provided by light-scattering experiments made by Doty *et al.* (1954) and Doty, Bradbury & Holtzer (1956), who also concluded that when the solvent was dichloracetic acid the polypeptide was in a solvated, random coil. Following this, Doty & Yang (1956, 1957) have measured the optical rotatory dispersion of poly-γ-benzyl-L-glutamate in ethylene dichloride,

dichloracetic acid and other solvents. They have also measured the optical rotation in mixtures of the first two solvents, and concluded that the extreme forms (α helix and random coil) of the polypeptide chain are obtained, respectively, in ethylene dichloride and dichloracetic acid. In a helical polypeptide with an asymmetric α carbon atom, as Cohen (1955) has pointed out, the 'form' rotation of the helix may either reinforce or diminish the rotation caused by the asymmetric centre. Fitts & Kirkwood (1956*a*, *b*) have calculated the specific optical rotation of an α helix without asymmetric residues and obtained a value $+132°$ for a right-handed helix of polyglycine in water. They consider the magnitude of the change in optical rotation on formation of the α helix found by Doty & Yang (which is in the positive direction when the α helix is formed) and conclude that their calculations are in agreement with these observations if the helices are assumed to be right-handed. Moffitt (1956*b*) has criticized the model on which these calculations are based and concluded, justifiably, that the numerical agreement is fortuitous. This is also evident from our earlier note (Elliott, Hanby & Malcolm 1956), in which we point out that the observed rotation in polyleucine is numerically different from that expected on the basis of Fitts & Kirkwood's calculations.

Huggins (1952) considers that left-handed α helices of L polypeptides are less stable than right-handed ones, because of the short distance (2·7 Å) between βC and O atoms in the left-handed helix. On the other hand, in a detailed examination of the stability of polypeptide folds with internal hydrogen bonds, Donohue (1953) has not found any strong reason why there should be a difference in stability between right- and left-handed α helices. Arndt & Riley (1955), in an investigation of the radial distribution function of α polypeptides and globular proteins, based on X-ray powder diffraction patterns, have concluded that they consist of α helices with the βC atom in Pauling & Corey's position 2 (1951). This corresponds to left-handed helices of L polypeptides (Pauling & Corey 1954). Brown & Trotter (1956) have examined the X-ray fibre diffraction patterns of well-oriented specimens of poly-L-alanine (Bamford, Brown, Elliott, Hanby & Trotter 1954) and have found that neither right-handed nor left-handed helices allow a good fit to be obtained between observed and predicted intensities, with the simplest unit cell which the data allow. They expressed a preference for the left-handed helix, and considered that a satisfactory fit might be obtained with some adjustment of the atomic co-ordinates. More recently, the same data have been re-examined (Elliott & Malcolm 1956) and it has been found that, with a 'statistical' unit cell containing randomly arranged parallel and anti-parallel helices the fit can be much improved; the right-handed form gives a far better fit of intensities than either the left-hand form or a mixture of both. Packing considerations strongly suggest that such a random arrangement is satisfactory, though they do not demand that the helices should necessarily be all either right- or left-handed. These conclusions are at variance with the findings of Arndt & Riley, and with the helix sense tentatively suggested by Brown & Trotter, but we believe them to be well-founded.

The X-ray observations do not altogether exclude the possibility that, in poly-L-alanine fibres, some left-handed helices are present, for if they were present in non-crystalline regions they might contribute to the strong layer-line streaks observed

in the diffraction pattern. However, it appears likely that these layer-line streaks are a direct consequence of the random up-and-down arrangement of the polypeptide chains, as is shown by some calculations which Dr Cochrane has kindly made available to us, and there is probably no need to postulate left-handed helices. We have recently (Elliott *et al.* 1956) described how measurements of optical rotation of a series of polyleucines (in benzene) of various D:L compositions show very clearly that only one sense of helix is present over a great part of the range. These and other measurements are reported below.

Optical rotation data are most easily discussed in terms of *residue* rotations, reduced to the value which they would have in a medium of unit refractive index (i.e. *in vacuo*). We propose the symbol $[R_{\text{vac.}}]$ for this quantity, rather than $[M]$, which is used by Fitts & Kirkwood (1956*b*) or $[m']$ as used by Moffitt & Yang (1956), to aviod confusion with the molar rotation, and to indicate explicitly that a correction for solvent refractivity has been applied. The relation between the specific rotation and the residue rotation $[R_{\text{vac.}}]$ is

$$[R_{\text{vac.}}] = \frac{\alpha R}{100} \frac{3}{n^2+2},$$

where R is the residue weight and n the refractive index of the liquid in which the polymer has a specific rotation α.

In an important series of papers, Moffitt (1956*a*, *b*) has developed the theory of optical rotation of an α helix, and Moffitt & Yang (1956) have shown that the anomalous rotatory dispersion observed by Doty & Yang for the α helix form of poly-γ-benzyl-L-glutamate is of the kind to be expected. The residue rotation $[R_{\text{vac.}}]_\lambda$ is given by the expression

$$[R_{\text{vac.}}]_\lambda = \frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2},$$

where a_0 is a constant which may be expected to vary with the nature of the side-chain of the polypeptide and to be dependent on solvation effects, whereas b_0 and λ_0 should be intrinsic properties of the helical skeleton; b_0 changes sign if the sense of the helix is changed. Moffitt & Yang find b_0 to be negative for poly-γ-benzyl-L-glutamate and poly-L-glutamic acid, and in good numerical agreement with the value which Moffitt (1956*b*) has calculated for a right-handed α helix. However, as will be shown later, the value of b_0 is not entirely independent of the side-chains, and this close agreement is perhaps fortuitous. Nevertheless, two independent techniques show that in simple α polypeptides the right-handed sense is dominant for the L-form, together confirming the absolute configuration of the amino-acid residue used in the interpretation of the X-ray data. It has also been shown that when the fraction of residues having the L-configuration (which we denote by L/(D + L)) exceeds 0·7, polyleucine in benzene exists entirely as right-handed helices.

In the work reported below measurements have been extended to other polypeptides and the various types of behaviour examined in detail.

Experimental

Synthesis of polypeptides

Polyleucine. Calculated quantities of the *N*-carboxy anhydrides of L- and DL-leucine were dissolved in dry benzene. Polymerization was initiated with tri-*n*-butylamine and allowed to proceed at 30° C.

The polyleucines were isolated by distilling off the solvent under reduced pressure and washing the residual polymer in methanol to remove traces of initiator. Finally, the polymers were obtained in a suitable state by freeze-drying from benzene solution.

Polyalanine. The *N*-carboxy anhydrides of L- and D-alanine were copolymerized in the required proportions at 50° C in benzene + dioxan (9:1) solution using tri-*n*-butylamine as initiator.

The polymers were filtered off, washed with ether and dried. (These polymers are of rather low molecular weight (d.p. ~ 50).)

Poly-α-amino-n-*butyric acid.* The required quantities of the D- and DL-*N*-carboxy anhydrides were copolymerized in dry nitrobenzene solution using benzylamine as initiator, the molar ratio of *N*-carboxy anhydride to initiator being 500. The isolation was carried out in the same way as for polyalanine.

Poly-γ-benzyl glutamate. The calculated quantities of the *N*-carboxy anhydrides of γ-benzyl-L- and DL-glutamate were copolymerized in dry dioxan using caustic soda dissolved in methanol as initiator. The polymers were isolated by precipitation with excess methanol. Finally they were dissolved in chloroform and cast as films on glass sheets.

Degree of polymerization. Although the copolymerization of the L- and DL-carboxy anhydrides was carried out, for each series of polymers, from the same batch of material using the same amount of initiator, it cannot be assumed that the degree of polymerization will be independent of the ratio L/(D + L). Traces of impurity capable of acting as initiators, if present in differing amounts in the L- and DL-anhydrides, would evidently cause the degree of polymerization to vary along the series of copolymers. Some information bearing on this possibility was obtained by measuring the specific viscosities of two different series of poly-γ- benzyl glutamates in dichloracetic acid, in which these polymers are in the random form. Specific viscosities (polymer concentration 0·5% w/v) are shown plotted against L/(D + L) in figures 5 and 6. From the measurements of Doty, Bradbury & Holtzer (1956) on viscosities of poly-γ-benzyl-L-glutamate it is seen that the enantiomorphs in our two series 373 and 375 have degrees of polymerization somewhat lower than 320 and 1860, respectively; these are the figures which would apply if our measurements of viscosity gave the values at zero polymer concentration. The viscosity falls greatly as the *meso* polymer is approached, and since all the polymers are in the random coil form in the solvent used for viscosity measurements, it is reasonable to suppose that the degree of polymerization falls likewise, though independent measurements of molecular weight are needed before the relation between viscosity and degree of polymerization is known for L and D copolymers. The α-amino-*n*-butyric acid copolymers show a similar fall in viscosity towards the *meso* polymer.

Measurement of optical rotation

Since the specific rotations of several polypeptides as reported in the literature are rather small, and moreover the solubilities in some cases are very low, it would have been difficult or perhaps impossible to have got satisfactory measurements with the usual form of visual polarimeter. We have accordingly employed a very sensitive photo-electric polarimeter of our own design (Malcolm & Elliott 1957) with which small rotations can be measured to about 0·001°.

The chief error in the measurements is caused by uncertainty as to polymer concentration, arising from the fact that it is sometimes hard to be sure that a sample has dissolved completely. Careful inspection eliminated most of this error, but occasionally it is difficult to be certain that a solution, before filtering, contains no gel. When necessary, the concentration was determined after filtering, by evaporation of a known volume of solution.

Most of the measurements were made with the light from a sodium lamp, except the dispersion measurements which have been made using a mercury lamp with suitable filters to isolate the stronger lines.

Results

Polyleucine

Infra-red spectra of solutions in benzene of poly-L-leucine and poly-DL-leucine (Elliott 1953) strongly suggest that both polymers have the α configuration. Curve *a* (figure 1) shows the residue rotation (corrected to unit refractive index) of the polymer in benzene for various values of L/(D + L). Since for the higher values of this ratio the polymer is not sufficiently soluble in pure benzene, different amounts of *m*-cresol were added (1 to 16 %) and the rotation in pure benzene was determined by extrapolation. The optical rotation plot is linear over a considerable range of

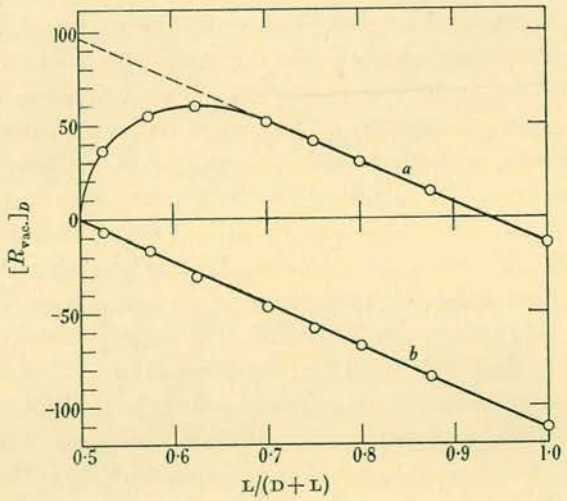

Figure 1. Optical rotation of copolymers of L- and D-leucine: (*a*) in benzene; (*b*) in trifluoracetic acid. Concentration 0·2 % w/v.

L/(D + L); this portion extrapolates to a value $[R_{vac.}]_D$ of 96°, which would be the optical rotation of a polypeptide with equal numbers of D and L residues if the linear relation held over the whole range. The linear part of the graph can mean only that over this range helices of one sign (right-handed in our case) persist; as the proportion of D residues increases, the negative contribution of the L asymmetric groups is cancelled out to an increasing extent (see discussion). When the fraction L/(D + L) is less than 0·7, the helices are no longer all right-handed. It must be supposed that either whole chains or parts of chains, because of local preponderance of D residues, become left-handed.

In trifluoracetic acid, the optical rotation is linear in L/(D + L), and the graph passes accurately through the point (0·5, 0). The rotation is therefore proportional to the excess of L over D residues; there is no 'form' optical rotation and the polypeptide chains must be in random configurations. We assume, as is usual, that the optical rotation of independent groups is additive. This assumption is supported by the straight-line graphs obtained for a variety of solvents and polymers.

The linear portions of the two graphs of figure 1 are parallel, hence the change in optical rotation on unfolding an α helix of poly-L-leucine (in benzene) to form a random coil (in trifluoracetic acid) is of equal magnitude but opposite sign to the rotation of a right-handed helix carrying equal numbers of D and L residues. This agreement appears to be a coincidence, and is not generally found with other polypeptides (see below).

Measurements of the dispersion of optical rotation of some of the polyleucines in benzene containing 5 % *m*-cresol (v/v) have been made at temperatures between 18 and 21° C, for values of L/(D + L) equal to 0·7, 0·75, 0·8 and 0·875. The addition of *m*-cresol to the benzene lowers the viscosity of the polymer solution and makes it easier to handle. It was found that at each wavelength the plot of L/(D + L) against the residue rotation was linear, showing that all the polymers were entirely in helical configurations of the same sense in this solvent. From the extrapolation of the linear plots, the values of the residue rotations for right-handed helices of the *meso* polymer were obtained at various wavelengths. Although these extrapolated values increase rapidly with diminishing wavelength over the range observed (unlike those for the individual polymers which have a maximum value at intermediate wavelengths) and appear superficially to correspond to normal dispersion, it is found that they cannot be represented accurately by a single Drude term. All the data, including the extrapolated values, can be made to fit Moffitt's formula for dispersion (see Introduction) with the same value of λ_0 which Moffitt & Yang obtained for poly-γ-benzyl-L-glutamate, namely, 2120Å. Figure 2 shows the data plotted in the way introduced by these authors. The values of b_0 from curves *a* to *e* are, respectively, -460, -446, -466, -470 and -513 degree cm²/decimole. These figures are appreciably smaller than those given by Moffitt & Yang for poly-γ-benzyl-L-glutamate (about -630 in the same units). There is, moreover, a trend towards larger values of b_0 as the polymers approach the *meso* composition. By giving λ_0 the value 2000Å, equally linear plots are obtained in which this trend is not apparent; b_0 is then approximately $-640°$. Our data do not allow an independent determination of λ_0 to be made, but it is evident that either (*a*) λ_0 is different in the two polymers, or (*b*) b_0 is different for

poly-γ-benzyl-L-glutamate and polyleucine and also (for polyleucine) is somewhat dependent on the D:L composition. It is interesting to note that, using a somewhat different procedure, Doty & Lundberg (1957), who also assumed λ_0 to be 2120Å, deduced that the value of b_0 for the right-handed *meso* form of poly-γ-benzyl glutamate in dioxan is about $-500°$. This is considerably less than the value for the enantiomorph but almost the same as our result for polyleucine.

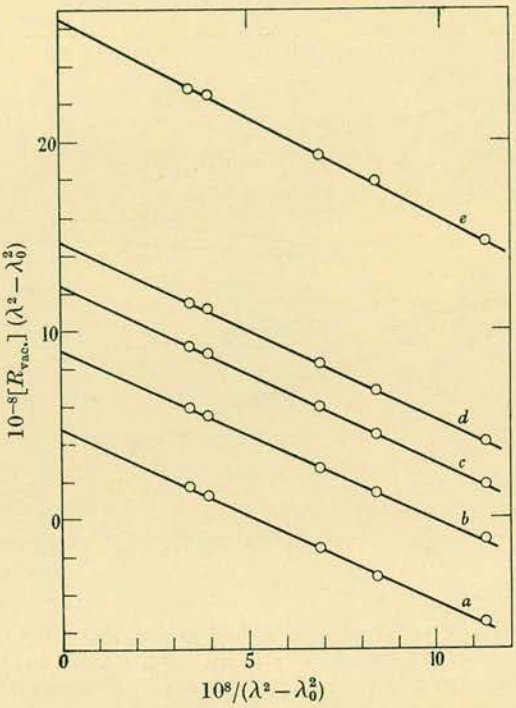

FIGURE 2. Dispersion of optical rotation of copolymers of L- and D-leucine in benzene containing 5% *m*-cresol (v/v). Polymer concentration 0·3% w/v, temperature 20° C. L/(D+L): (*a*) 0·875; (*b*) 0·80; (*c*) 0·75; (*d*) 0·70; (*e*) 0·50 (by extrapolation). λ in ångströms.

Polyalanine

Plots of $[R_{vac.}]_D$ against L/(D+L) for six solvents are given for polyalanine in figure 3. Only the two polymers nearest the *meso* form were soluble in water, and in this solvent the rotation is practically identical with that in trifluoracetic acid. Evidently in phosphoric and trifluoracetic acids, in water and in aqueous lithium thiocyanate (saturated at 20° C) the configuration is random, and large solvation effects must be present, for the rotation in the lithium salt solution is much less than in the other solvents. Yang & Doty (1957) have found evidence of such effects in poly-γ-benzyl-L-glutamate from consideration of the dispersion of rotation.

Polyalanines are not soluble in non-polar solvents, and in order to obtain evidence of the helical configuration we have used a very low concentration of polymer (0·05% w/v) in chloroform containing 1% of dichloracetic acid (v/v). The polymer was first dissolved in pure dichloracetic acid and chloroform was then added. The plot shows a maximum rotation when L/(D+L) is about 0·9, but the linear part

so characteristic of polyleucine in benzene has almost completely disappeared. A rough value for the residue rotation of polyalanine in a right-handed helix carrying equal numbers of D and L residues is got by drawing the broken line tangent to the right-hand end of the graph and taking the intercept on the $[R_{vac.}]$ axis. As will be shown in the discussion, the form of the graph corresponds to chains whose configuration is mainly α helix, but with varying amounts of right- and left-handed forms.

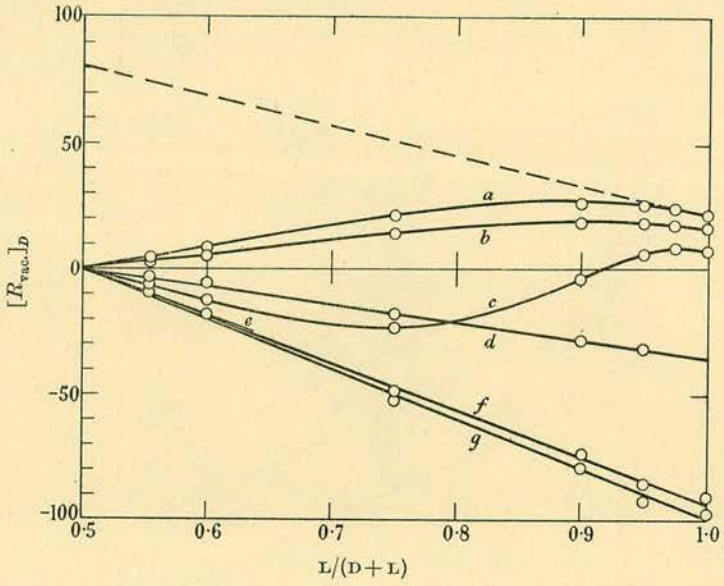

FIGURE 3. Optical rotation of copolymers of L- and D-alanine at 20° C. (*a*) Concentration of polymer 0·05 % w/v in chloroform containing 1 % dichloracetic acid (by volume). (*b*) Concentration of polymer 0·5 % w/v in chloroform containing 10 % dichloracetic acid (by volume). (*c*) Concentration 0·2 % w/v in dichloracetic acid. (*d*) Concentration 0·2 % w/v in saturated aqueous lithium thiocyanate. (*e*) Concentration 0·2 % w/v in water. (*f*) Concentration 0·2 % w/v in trifluoracetic acid. (*g*) Concentration 0·2 % w/v in phosphoric acid.

The curve obtained in dichloracetic acid is unusual, the rotation changing from negative to positive as L/(D + L) increases. The shape of this curve is sensitive to changes in molecular weight of the polymer; the highest molecular weight poly-L-alanine which we have examined has an $[R_{vac.}]_D$ value + 17½°, considerably greater than that for the enantiomorph on figure 3. This curve (for reasons given later) shows that polyalanine in dichloracetic acid is randomly coiled for L/(D + L) less than about 0·6, and as this fraction increases, increasing amounts of helix are found, with right-handed predominating over left-handed forms. This interpretation of optical rotation data agrees remarkably well with some deductions which have been made by Bamford, Elliott & Hanby (1956). Poly-D-, poly-L- and poly-DL-alanine were prepared under the same conditions of initiation, and from amino nitrogen analysis the degrees of polymerization were found to be 150, 150 and 130, respectively. The limiting viscosity number of the *meso* polymer was found to be very much lower than those of the enantiomorphs, suggesting that the molecules of the *meso* polymer are

more randomly coiled and less rigid. It seems likely that poly-L-alanine of sufficiently high molecular weight would all be in a right-handed helical form in dichloracetic acid even at low concentration (contrast with other polymers).

Some evidence was found that polyalanines are degraded by warm phosphoric acid, and all manipulation in this solvent was carried out at room temperature.

*Poly-α-amino-*n*-butyric acid*

Results for poly-α-amino-*n*-butyric acid are shown in figure 4. For convenience in the preparation, the D-amino acid was present in excess. To make comparison with other polypeptides simpler, the results in figure 4 and in table 1 are given as if the L-amino acid were the main constituent. The behaviour is different from that of polyalanine, for in dichloracetic acid the polymer is in the random form for all values

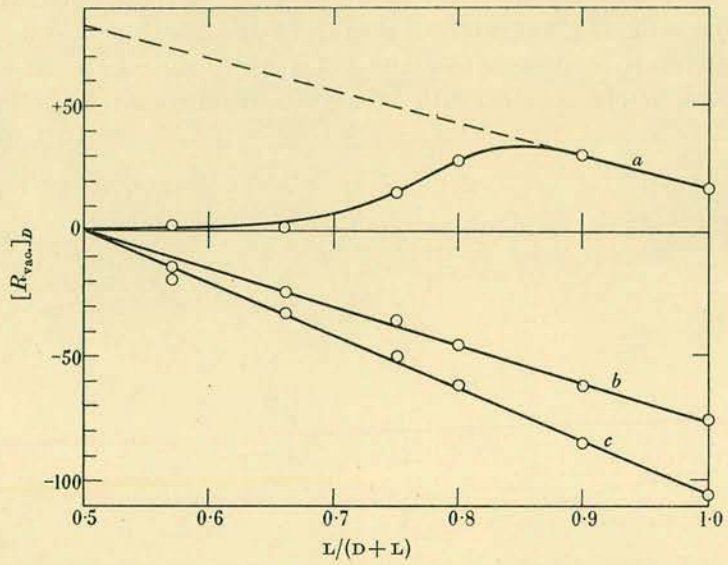

FIGURE 4. Optical rotation of copolymers of L- and D-α-amino-*n*-butyric acid at 20° C. (*a*) Concentration of polymer 0·5 % w/v in chloroform containing 10 % v/v dichloracetic acid. (*b*) Concentration 0·2 % w/v in dichloracetic acid. (*c*) Concentration 0·2 % w/v in trifluoracetic acid.

TABLE 1. RESIDUE ROTATIONS (CORRECTED TO UNIT REFRACTIVE INDEX) FOR RIGHT-HANDED α HELIX CARRYING EQUAL NUMBERS OF D AND L RESIDUES

polypeptide		$[R_{vac.}]_D$
polyalanine		+ 82° (chloroform + 1 % dichloracetic acid)
poly-α-amino-*n*-butyric acid		+ 81° (chloroform + 10 % dichloracetic acid)
polyleucine		+ 96° (benzene)
poly-γ-benzyl glutamate	prep. no. 373	+ 75° (dioxan*, chloroform†, pyridine)
		+ 68° (dimethylformamide, *m*-cresol)
	prep. no. 375	+ 70° (chloroform)
		+ 67° (dioxan)
		+ 65° (pyridine)
		+ 58° (*m*-cresol)
		+ 54° (dimethylformamide)

* By a different method (Doty & Lundberg 1957) the value + 90° has been found in dioxan.

† Using the same method, Blout, Doty & Yang (1957) have obtained the value + 78°.

of L/(D + L) at the concentration employed. For evidence of the helix, it was found necessary to use a chloroform mixture containing 10 % by volume of dichloracetic acid; the more nearly the composition approaches that of the *meso* form the less soluble is the polymer. The linear part of the graph, though short, is quite appreciable. The shape corresponds to a considerable amount of random-coil form at values of L/(D + L) near 0·5.

Poly-γ-benzyl glutamate

Poly-γ-benzyl-L-glutamate has been extensively studied by Doty, Blout and others, and because of its solubility in a variety of liquids it is very suitable for examination of the relation between molecular configuration and solvent. Yang & Doty (1957) have concluded that this polymer is in a helical form in *m*-cresol, chloroform, ethylene dichloride, dimethylformamide and dioxan, and in a random coil in dichloracetic acid, trifluoracetic acid and hydrazine.

The optical rotations of two series (nos. 373 and 375) in a number of solvents are shown in figures 5 and 6, together with the specific viscosities at 0·5 % w/v in dichlor-

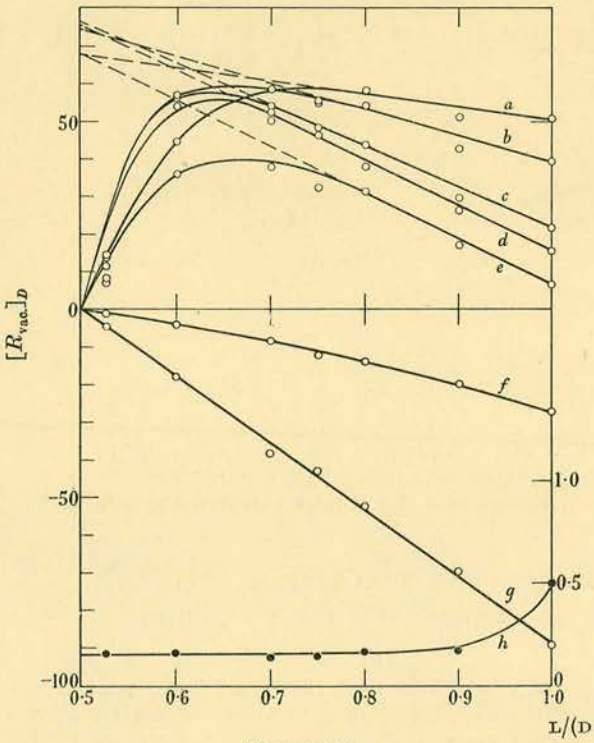

FIGURE 5

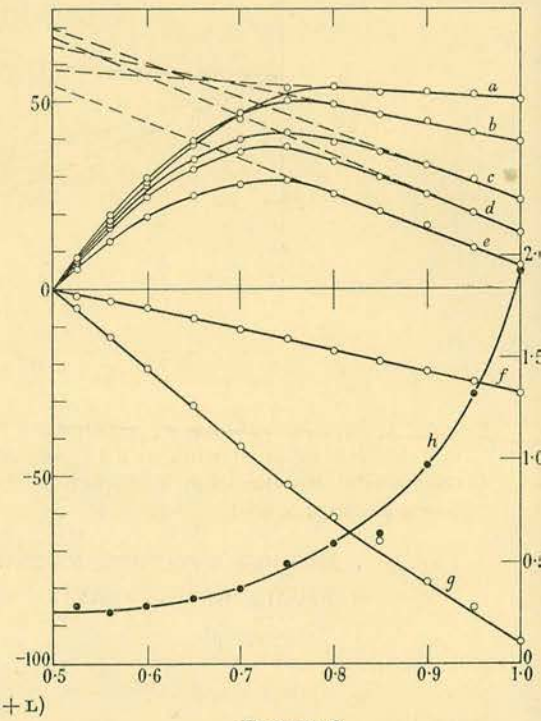

FIGURE 6

FIGURE 5. Optical rotation of copolymers of L- and D-γ-benzyl glutamate (series 373) in various solvents at 20° C. Concentration 0·5 % w/v. (*a*) *m*-cresol; (*b*) pyridine; (*c*) chloroform; (*d*) dioxan; (*e*) dimethylformamide; (*f*) dichloracetic acid; (*g*) trifluoracetic acid. Curve *h* shows specific viscosity at 0·5 % w/v in dichloracetic acid (25° C).

FIGURE 6. Optical rotation of copolymers of L- and D-γ-benzyl glutamate (series 375) in various solvents at 20° C. Concentration 0·5 % w/v. (*a*) *m*-cresol; (*b*) pyridine; (*c*) chloroform; (*d*) dioxan; (*e*) dimethylformamide; (*f*) dichloracetic acid; (*g*) trifluoracetic acid. Curve *h* shows specific viscosity at 0·5 % w/v in dichloracetic acid (25° C).

acetic acid. The results are in agreement with Yang & Doty's conclusions, showing that the enantiomorph is either wholly in right-handed α helices or wholly in the randomly coiled form. The wide range of values of $[R_{\text{vac.}}]_D$ for the L polymer in different solvents must therefore be attributed to the existence of large solvation effects, which have been inferred by Yang & Doty on other grounds. The value of $[R_{\text{vac.}}]_D$ obtained by extrapolating the linear part of the curve to $\text{L}/(\text{D}+\text{L}) = 0{\cdot}5$ (which corresponds to the rotation of the *meso* polymer in a right-handed helix) shows much less dependence on the solvent.

Although poly-γ-benzyl-L-glutamate in dichloracetic acid is in a randomly coiled form at low concentration, at higher concentration it goes over into the helical form. This is shown by the change from negative to positive rotation which takes place when the concentration is raised from 15 % w/v to about 23 % w/v. At or near the latter concentration, the birefringent form begins to separate out (Robinson 1956, 1957) at room temperature, and optical rotation measurements become impossible. However, when the temperature of the polymer solution is raised the birefringence disappears and the rotation is found to be positive, with a negative temperature coefficient.

Discussion

There are some slight differences between the two sets of observations on poly-γ-benzyl glutamate which call for comment. The optical rotations of the two L polymers agree within experimental error in all the solvents used, in spite of the considerable difference in molecular weight, and it therefore seems likely that over the linear portion of the curves the optical rotation is not sensitive to changes in the degree of polymerization. The straight parts of the graphs for series 373 in those solvents where α helices are present have consistently greater slopes, and extrapolate to higher values of $[R_{\text{vac.}}]_D$ for the helical *meso* polymer, than do the corresponding curves for series 375; in solvents where the polymers exist as random coils, series 375 gives slightly larger rotations than series 373, the difference being least for the *meso* polymers and the L enantiomorphs. We think it likely that these discrepancies are attributable to slight involuntary variations in the polymerization procedure. It is quite possible that steric factors may affect reaction rates sufficiently to cause the D:L composition of the polymer to be slightly different from that of the mixture of *N*-carbonic anhydrides, and also to cause the arrangement of D and L residues in the chain to be not entirely random; as we have already seen, there does appear to be an unexpected dependence of molecular weight on D:L composition. It is not unreasonable to suppose that these effects occur systematically in a series of polymers prepared at the same time from the same materials, but may operate to different degrees in different series. Comparison of figures 5 and 6 also shows that the single sense of helix persists rather further towards the *meso*-composition in the case of the 373 series. This is contrary to what one would expect if there were no breaks in the helix, for in that case the single sense should be favoured by increase in molecular weight. A possible explanation is that in the 375 series there is a greater tendency for the L and the D residues to occur in runs, which would make occasional reversal of the helix sense energetically more profitable, or alternatively for whole molecules to deviate more widely from the mean D:L composition.

It must be emphasized that the explanations advanced in the two previous paragraphs are only tentative; confirmation must wait upon the careful study of the copolymerization reaction and further physical measurements on more completely characterized polymers which are the subject of current investigations in this laboratory.

It is convenient to consider the optical rotation of a polypeptide in an α helix as having two components—the form rotation of the helical skeleton, and the contribution of the solvated side-chains. It might be thought that side-chains associated with L and D residues of the same amino-acid on a helix of a given sense would cause equal and opposite rotations, but this is not necessarily so. On a polypeptide helix of a given sense, the L and D configurations of a residue (corresponding to βC_1 and βC_2 for a right-handed helix) are not mirror images, since the helix sequence is polar. The consequence of this polarity is that on a right-handed helix the βC atom of an L residue is in the *trans* position with respect to the oxygen atom of the neighbouring carbonyl group, whereas that of a D residue is in the *cis* position. The contributions of L and D residues could therefore be of quite different magnitude, and may even have the same sign. From the slopes of the straight parts of the curves of optical rotation against L/(D + L) we can therefore determine only the algebraic difference between the contributions of L and D residues. It will be seen that in all the cases so far studied, the D residue contribution is algebraically greater.

When poly-γ-benzyl glutamates are dissolved in *m*-cresol, the linear portion of the curve is nearly horizontal, indicating that the contributions of solvated L and D residues are almost identical. Yang & Doty (1957) have remarked that the dispersion characteristic of polypeptides in the α helix form is exhibited to an exaggerated degree by the L enantiomorph in this solvent, and have suggested that this is due to the formation of a more ordered helical arrangement of solvated side-chains which give rise to a helix-type rotation additional to that arising from the backbone. There is, however, a simpler explanation. We have seen above that the contributions from the solvated L and D residues (in *m*-cresol) must be nearly equal; if they are both close to zero we should expect to observe an enhanced helix-type dispersion, for the optical rotation of the solution would then be due almost entirely to the form rotation of the helix, without the effects which in most other cases are superimposed upon it, and which tend to modify the helix dispersion.

If this explanation be correct, we would expect the optical rotation of the *meso* poly-γ-benzyl glutamate in *m*-cresol (obtained by extrapolation) to be fairly close to the value for an unadorned α helix. The corresponding values for other solvents are all close to that for *m*-cresol, while the optical rotations of the L-enantiomorph show a much greater dependence on solvent. It seems therefore that there is in fact a cancellation of the effect of the L residues and their attendant solvent molecules as we move towards the *meso* polymer, so that the contributions from L and D residues must be of opposite sign and comparable magnitude. This conclusion is confirmed by an examination of the dispersion of the helical *meso* polymer in dioxan solution obtained by Doty & Lundberg (1957). This is of the same exaggerated form observed for the enantiomorph in *m*-cresol.

In the case of other polypeptides the restricted range of solvents allows us to use

only the argument from the dispersion of the *meso* polymer. For polyleucine in benzene containing 5 % *m*-cresol we are led to a similar conclusion to that reached for poly-γ-benzyl glutamate.

We should expect the optical rotation of a *meso* polymer with a single sense of helix to be independent of the nature of the solvent and the side-chains only if the contributions of the solvated side-chains cancelled completely in all cases. The variation observed from polymer to polymer and solvent to solvent is probably due in part to the varying extent to which cancellation occurs.

In solvents in which α helices occur, the observed rotation in the non-linear part of the L/(D + L) graph is less than it would be if all the polymer molecules were in the right-handed helical form. This could be caused either by admixture of left-handed helical forms, or by partial destruction of the α helix to form random configurations. Since, as Doty & Yang have shown, the dispersion of the rotation for the α helix form of an L polypeptide is 'anomalous', and distinguishable at sight from the normal dispersion of a random coil, it might be thought that there would be no difficulty in distinguishing between the two possibilities. In fact, however, it is difficult, if not in practice impossible, to distinguish by dispersion measurements a mixture of equal numbers of right- and left-handed helices from random coils. This is easily seen by considering the dispersion formula of Moffitt & Yang (above). If b_0 changes only in sign for a change in helix sense, then the second term in the expression vanishes when we have equal numbers of right- and left-handed helices, and the dispersion is normal. It is true that λ_0 will have the value for the helix, but although the corresponding constant for a random coil could be different, the difference should be small because both dispersions depend on the peptide absorption band. In fact, Yang & Doty (1957) appear to have obtained the same value for λ_0 for a random coil as Moffitt & Yang (1956) found for the α helix (2120 Å). If both senses of helix are present in different amounts, only the excess of the predominant species will contribute to the anomalous dispersion. It does not seem possible, therefore, to distinguish between the two possibilities by this means. For the same reasons, conclusions about the absence of two helix senses in proteins must depend on other considerations.

The following considerations throw some light on the interpretation of the non-linear part of the L/(D + L) curve. In going from the pure enantiomorph to the *meso* form of a simple polypeptide in a not-too-polar solvent, two distinct transitions may occur. If the α helix form is considerably more stable than the random-coil configuration, then the effect is simply to increase the proportion of helices with a sense opposite to that found for the enantiomorph; the *meso* compound itself will of course contain equal numbers of both senses. If, however, the excess free energy per residue of the random coil form (of the enantiomorph) is of the same order as the free energy of strain caused by placing a residue on a helix of the 'wrong' sense, then for polymers close to the *meso* composition the random coil will be of comparable stability to the α helix, and indeed may even be the more stable form. In this case the passage from enantiomorph to *meso* form will be marked by an uncoiling of the α helix. As we have already seen, dispersion measurements do not in practice enable us to distinguish between these two effects, but an examination of the L/(D + L) curve does

enable us to make a qualitative estimate of the relative importance of the two processes.

The argument is perhaps most easily followed if we replot the data in a slightly different manner. In figure 7, AB is drawn parallel to the linear part of the graph, and thus represents the contribution to the rotation of the helical arrangement of asymmetric centres and their attendant solvent molecules. If, therefore, we plot as ordinates the height of the experimental points above AB, we obtain a curve which shows how the 'form' rotation of the helix varies with change in L/(D + L), and this gives an indication of the excess of right-handed over left-handed helices at any composition.

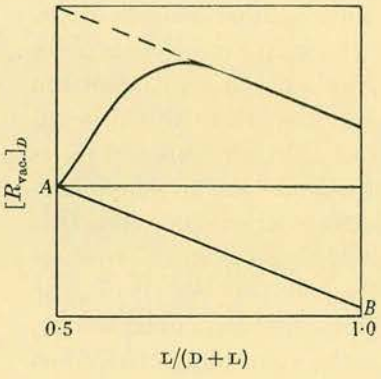

FIGURE 7

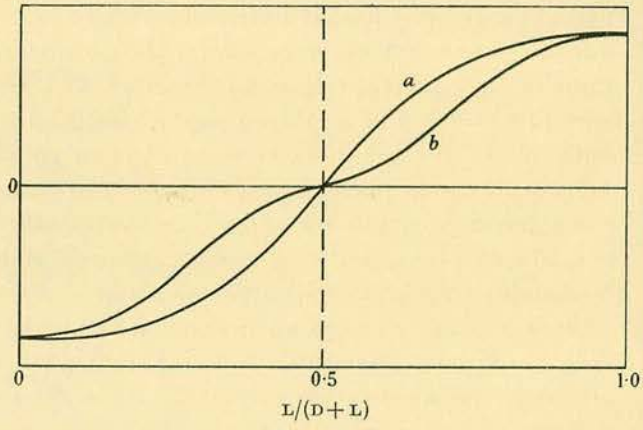

FIGURE 8

FIGURE 7. Hypothetical graphs showing total rotation (upper curve) and contribution from solvated asymmetric groups for an α helix (lower curve).

FIGURE 8. 'Form rotation' of α helices: (*a*) helical form predominant at all values of L/(D + L); (*b*) random coil predominant for the *meso* polymer.

When the α helix is always the stable form, we have a single continuous transition from left-handed helices when L/(D + L) = 0 to right-handed helices when L/(D + L) = 1, and the curve will be of the form shown at *a* in figure 8. With the simplest possible model, the curve is a hyperbolic tangent; complicating factors may modify it but can hardly alter its general shape. This curve has only one inflexion, at L/(D + L) = 0·5, when the gradient is a maximum.

When the random coil is the stable form for the *meso* polymer, we have in contrast two transitions; when L/(D + L) is less than 0·5 we have an equilibrium between left-handed helices and random coils, whereas when L/(D + L) is greater than 0·5 the equilibrium is between random coils and right-handed helices. In this case, therefore, each half of the range of L/(D + L) values is occupied by a curve of the same form as *a*, and the whole curve is as shown at *b* in figure 8. This curve has *three* inflexions, that at L/(D + L) = 0·5 occurring when the gradient is a minimum. It is clear that the two extreme types of behaviour lead to quite different forms of curve, and may therefore be distinguished experimentally. While it is not possible to estimate quantitatively the relative importance of the two processes in any system, we can see which is

predominant. In particular, the existence of positive curvature in the right-hand part of the curve is a certain indication that the random coil form is in excess in the *meso* compound.

It has been assumed in the preceding discussion that the enantiomorph is entirely in helices of one sense. The arguments are not affected by the occurrence of a proportion of random coils or 'wrong' helices when L/(D + L) = 0 or 1, but in such a case it is not of course possible to make more than an estimate of the line AB. However, variations of slope in this line cannot produce or destroy the inflexion, so that the existence of large amounts of random coil may still be inferred.

We are now in a position to interpret the curves obtained with polyalanine (figure 3) and poly-α-amino-n-butyric acid (figure 4). In figure 3, the curve c (dichloracetic acid) would clearly have three points of inflexion if the whole range of L/(D + L) were considered. The configuration of the polymer is therefore mainly random coil for the *meso* form, and mainly α helix for the enantiomorph. Curves a and b, on the other hand, show that at all values of L/(D + L) the helix form is in excess in the chloroform-dichloracetic mixtures used, the proportion of left-handed helices being greatest for the *meso* composition. The relatively small curvature of the part of the curves rising from the *meso* point, compared with the great negative curvature in curve a for polyleucine (figure 1) suggests, however, that a significant amount of the random coil form is present for near *meso* compositions.

Poly-α-amino-n-butyric acid (figure 4a) in chloroform containing 10 % dichloracetic acid shows behaviour intermediate between the two cases just discussed. Detailed comparison between different polypeptides would be justified only if the molecular weights and molecular-weight distributions were known to be similar.

Where both senses of α helix are present we cannot from optical rotation measurements say whether there is a mixture of right-handed helical chains with left-handed ones, or whether there are frequent changes of helix sense along each chain. Since a reversal of sense requires the breaking of at least three hydrogen bonds, it might be expected, on energetic grounds, that such changes would be frequent only if the strain energy associated with a residue on the 'wrong' sense of helix was considerable. While such strain is certainly appreciable, its significance has been questioned (Donohue 1953). Wippler (1956) has found that poly-DL-phenylalanine in benzene behaves as if the molecules were flexible, which would be expected if a change of helix sense occurred from place to place. We have tried to obtain some further information from the infra-red spectra of polyleucines at a concentration of 1 % w/v in carbon tetrachloride. The frequency of the hydrogen-bonded NH groups is at about 3300 cm^{-1}, whereas unbonded NH groups absorb at 3460 cm^{-1}. The ratio of intensity of these two bands in poly-DL-leucine was greater than 1600:1. Although the molar absorption coefficient of the bonded NH band is probably considerably greater than that of the free NH band, the great difference between the observed intensities means that there are not many free NH groups, and indeed the free NH absorption might perhaps be entirely accounted for by the unbonded groups at the ends of the α helices. Though the possibility of intermolecular association cannot be ruled out, the results do at least suggest that frequent changes of sense do not occur.

The dependence of chain configuration on concentration, which we have observed in poly-γ-benzyl-L-glutamate solutions in dichloracetic acid, is of considerable interest, and suggests that such an effect might occur with other polypeptides in polar solvents. In particular, it may be that the configuration of some proteins in aqueous solution might be sensitive to concentration, and that the intra-molecular arrangement in protein crystals could be different from that of the same protein in dilute aqueous solution.

We are grateful to Professor Paul Doty for having made many of his results available to us in advance of publication.

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STRUCTURE AND PROPERTIES OF SYNTHETIC POLYPEPTIDES AND SILK PROTEINS

Infra-Red Studies of Polypeptides Related to Silk

STRUCTURE AND PROPERTIES OF SYNTHETIC POLYPEPTIDES AND SILK PROTEINS

INFRA-RED STUDIES OF POLYPEPTIDES RELATED TO SILK

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Infra-red spectra and X-ray powder photographs of regenerated, water-soluble forms of the silk spun by *Anaphe moloneyi* reveal the presence of α-helical configurations when the regenerating agent is trifluoracetic acid. When the water-soluble material is freeze-dried from aqueous solution, the characteristic diffraction pattern of the α-helix is not found. Spectra of the oriented, β-form of the silk have been observed; the specimens were made by rolling the water-soluble form.

Infra-red spectra and X-ray powder photographs of two molecular configurations of polyglycine have been made and their significance is discussed.

The existence of a folded form of polypeptide chain is now supported by much experimental evidence obtained from an examination of X-ray diagrams and infra-red spectra of synthetic materials. For materials which have been examined in a well-crystallized, oriented form (poly-γ-benzyl-L-glutamate, poly-γ-methyl-L-glutamate, poly-L-alanine) the X-ray evidence is very strongly suggestive of a helical fold of the kind proposed by Pauling, Corey and Branson.[1] The

subject has recently been reviewed by Crick [2] and need not be considered here in detail. The α helix of Pauling, Corey and Branson was arrived at from considerations of conditions for minimum energy in a folded polypeptide chain. These considerations exclude many folds which are sterically possible and suggest that the α helix is the most stable form of fold with internal hydrogen bonds.

Many synthetic polypeptides have been investigated in the Courtauld laboratories here and in Coventry, and the indications have been, until lately, that only two forms of chain occurred (according to the method of preparation), namely, the α-helix type and the extended β-chain type. It will be shown later that polyglycine precipitated from solution is exceptional. The X-ray diagram of this modification, in an impure form, was obtained by Meyer and Go,[3] but it has apparently not previously been considered as an exception. The question of the stability of some other folds has been examined by Donohue [4] and by Low and Baybutt,[5] as a result of which it appears that the stability of these folds may not be greatly inferior to that of the α helix. It has, of course, been widely realized that in proteins, as distinct from polypeptides, factors may be present which would favour configurations other than the α helix.

The infra-red spectra of α-helix polypeptides are characterized by a carbonyl stretching mode in the region 1652-1657 cm^{-1} for optically active polypeptides, and a slightly higher range for *meso* forms.[6] The wave number of this band only changes by about 2-3 cm^{-1} on going from the solid form to a *ca.* 1 % solution in an inert solvent, and there is no reason for doubting that the folded form is preserved in these conditions. Direct evidence on this point has been given by Doty, Holtzer, Bradbury and Blout.[7] The synthetic α-helix polypeptides pack (at least approximately) hexagonally, and it is possible to get a rough idea of the $10\bar{1}0$ spacing in a particular case from the measured density, or from considerations based on van der Waals' radii, etc. With this information, the presence of an α helix may be recognized by the occurrence of a (relatively) strong ring of the appropriate diameter on the powder photograph.

It will be seen from the foregoing that two criteria may be applied to recognize α helices in unoriented polypeptides, one based on the frequency of the C=O mode, the other on the presence of a dominant ring in the X-ray powder photograph. If orientation can be produced then the characteristic infra-red dichroism[8] and X-ray fibre pattern may also be sought.

The problem of the existence of a regular type of fold in crystalline or globular proteins has received much attention in recent years, and after the establishment of good evidence of the presence of α helices in synthetic polypeptides, it was natural to look for evidence of the same fold in proteins. The postulates from which Pauling and Corey deduced the α helix contain nothing to suggest that this will not be the stable form in proteins, and indeed they claimed to have found evidence that such a helix occurred in haemoglobin.[9] However, the parallel rod-like arrangement seen in Patterson diagrams of some crystalline proteins, as Crick [10, 11] has pointed out, should be much more marked if parallel α helices were present, though the data for haemoglobin are not incompatible with α helices if the molecules are not parallel, and Carlisle, Scouloudi and Spier,[12] in a detailed examination of ribonuclease, have concluded that there is insufficient evidence for the presence of α helices in this protein. The low infra-red dichroism in this protein gives no cause for belief that they are made up of α helices.[13]

Since in crystalline proteins the range of side-chain size is great, and in addition active side chains are present, it is perhaps likely that α helices, if present, would be so distorted as to be difficult to recognize. For this reason, a protein such as silk, which consists largely of the residues of the small amino acids, glycine and alanine, has advantages. The molecular weight is high and there are apparently no cross-links, so the properties may be expected to resemble the synthetic polypeptides, about whose configuration much is already known. The very low content of ionizable side-groups in silk ensures that infra-red bands from ionized

COO^- groups will not be present to any appreciable extent; such bands may, in certain cases, appear in protein spectra and may be confused with bands from the peptide groups.[14, 15]

Water-soluble silk, obtained either by dilution of the contents of the silk gland or by regeneration of silk fibroin, has been known for some time and evidence has been produced that it exists in a folded form.[16] The configuration of silk fibroin (which is, of course, not water-soluble) is known to approximate to a fully extended polypeptide chain. Recently, the work of Lucas, Shaw and Smith [17] at the Shirley Institute has shown that the proportion of amino-acid residues with large side-chains is particularly low in the silk of the African moth *Anaphe moloneyi*, and that this material is a fair approximation to an alanine-glycine co-polymer. We shall be concerned in this paper with the question of the chain configuration of water-soluble Anaphe silk, and with that of related materials.

EXPERIMENTAL

The infra-red spectra were recorded on a single beam Grubb-Parsons spectrometer with rock-salt prism and a Schwarz thermocouple. Polarized radiation, when used, was obtained by inserting a selenium transmission polarizer of six films in the beam.[18] The specimen was carried on a precision rocking mechanism and moved in and out of the beam at 2-sec intervals, to record incident and transmitted intensities.

WATER-SOLUBLE *Anaphe moloneyi* SILK

Anaphe moloneyi silk is not soluble in aqueous lithium bromide, but dissolves readily in trifluoracetic acid. If cast from this solvent on a mercury surface and air-dried, a film may be obtained which is completely water-soluble. However, the process does not always result in a soluble film, and a more certain method is to dissolve the silk in a small quantity of trifluoracetic acid, to which an excess of water is added. This is then frozen and drying is carried out by evaporation in a good vacuum. The resulting material is water-soluble. Specimens of freeze-dried Anaphe silk were made by redissolving this material in water and again freeze-drying on a support of thallium bromo-iodide. The specimen was quickly transferred to a small cell containing phosphorous pentoxide, which was then mounted on the rocking mechanism.

The extended, oriented form of Anaphe silk was obtained in a form suitable for observation by casting on silver chloride from a solution in trifluoracetic acid, air drying and then rolling in a jeweller's rolling mill. It appears best to carry out the rolling before the solvent has entirely evaporated; the degree of orientation obtainable is high but not readily reproducible. The silver-chloride film was dissolved off in a solution of sodium thiosulphate, since interference bands produced by a thin film of silver chloride are troublesome.

POLYGLYCINE

Oriented films of β-polyglycine were made in a manner analogous to those of Anaphe silk from a solution of the polymer in dichloracetic acid. Similar spectra are obtained whether the polymer is cast from dichloracetic acid or trifluoracetic acid but a completely different spectrum is obtained if the polymer is precipitated from solution. We shall call the β form (obtained by casting) polyglycine I, and that obtained by precipitation polyglycine II. In general, precipitation produces predominantly form II but usually with a proportion of form I. The spectrum shown was obtained from a specimen prepared by precipitation with water from a 1 % solution of polymer in saturated aqueous lithium bromide solution. It was found that the best way to obtain a specimen without any trace of form I was to add, with thorough mixing, as much water as possible (in this case about an equal volume) without the polymer precipitating out, and then to pour this mixture into an excess of water. The precipitate was then washed and centrifuged several times to remove all lithium bromide. Similar spectra were obtained by precipitation with water from a solution of polymer in dichloracetic acid. No change in the spectrum has been produced by heating the precipitate to 170° C in a good vacuum for 1 h or subjecting it to a high pressure (approximately 20 tons in.$^{-2}$). The structure is therefore unlikely to be a hydrated form and is quite stable. It is soluble only in the solvents which dissolve polyglycine I. In this respect it differs from other synthetic polypeptides which have markedly different solubilities depending on whether they are in the α or β form.

By adjusting the conditions of precipitation it was possible to control the particle size. In this way an aqueous suspension of particles dried on a thallium bromo-iodide substrate was obtained sufficiently finely divided for scatter of radiation to be inappreciable in the infra-red. This avoided the use of mulls.

DISCUSSION

POLYGLYCINE

Spectra of polyglycine which have appeared in previous publications have shown some variations;[19-23] it now appears possible that this may be a consequence, at least in part, of the existence of two modifications of polyglycine, which as may be seen from fig. 1, have different spectra. Polyglycine I is a typical

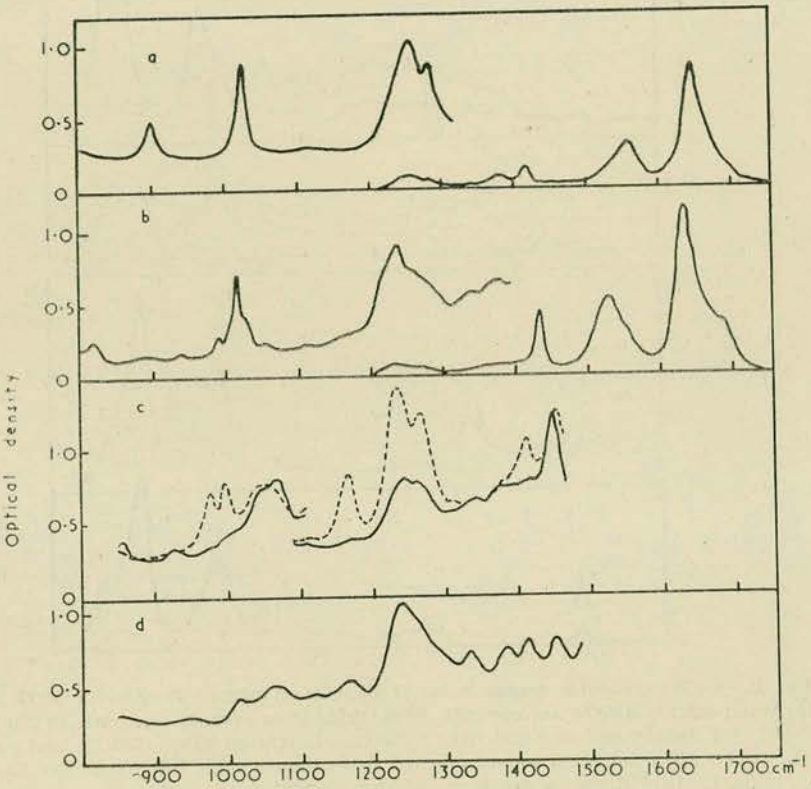

FIG. 1.—(*a*) Polyglycine II, precipitated by water from solution in aqueous LiBr. (*b*) Polyglycine I, the same specimen as (*a*), cast from dichloracetic acid. (*c*) *Bombyx mori* silk gut. Full line, *E* vector perpendicular to fibre axis; broken line, *E* vector parallel to fibre axis. (*d*) Water-soluble Bombyx silk (from solution in aqueous LiBr).

β spectrum, with the NH stretching mode at 3300 cm^{-1}, the C=O mode at 1632 cm^{-1} and the complex band which arises partly from NH deformation at 1521 cm^{-1}. Fig. 2*d* shows that the peptide bands in polyglycine I have the usual dichroism found in oriented β structures. It may be recalled that a rather strong band which appears at *ca.* 1690 cm^{-1} in polyglycines of low molecular weight was attributed to small peptides.[6] The polyglycine used in the work now reported was free from molecules of low molecular weight, and it appears that there is a band (of considerably reduced intensity) at 1685 cm^{-1} which is really attributable to polyglycine. It shows parallel dichroism, and in this respect resembles a very weak band which appears at 1695 cm^{-1} in the spectrum of β-poly-L-alanine,

but not in α-poly-L-alanine (Elliott,[23] fig. 2). A band at about the same wave number appears in other β polypeptides, and in silk.

Polyglycine II is interesting in having a C=O band at 1648 cm^{-1}, considerably higher than normal β frequencies but lower than the range of frequencies found in α polypeptides. The NH stretching mode is at 3290 cm^{-1} and the weak band which usually accompanies NH stretching modes, at 3095 cm^{-1}, is much stronger than in any synthetic polypeptide which we have examined. The NH deformation

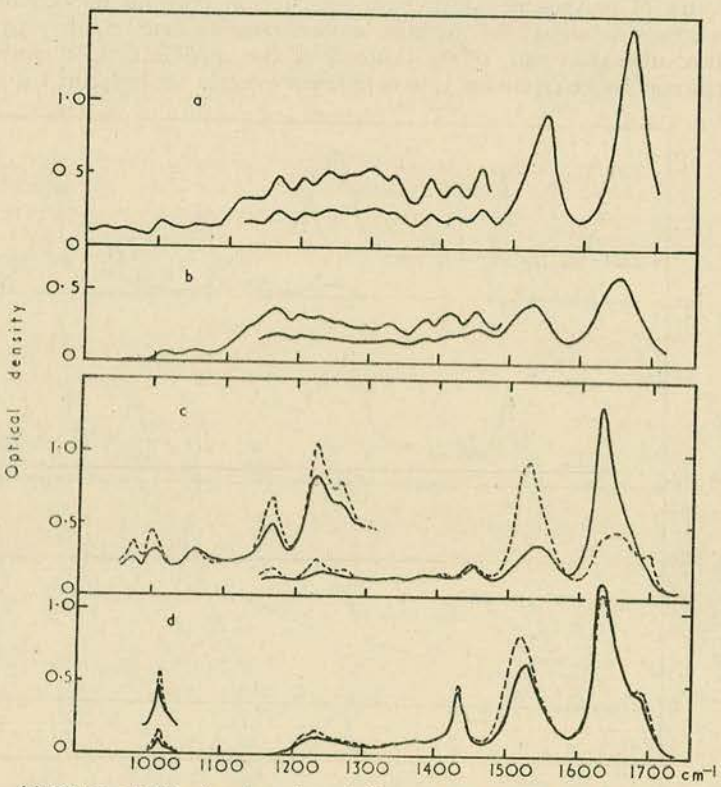

FIG. 2.—(*a*) Water-soluble *Anaphe moloneyi* silk, cast on mercury from trifluoracetic acid. (*b*) Water-soluble *Anaphe moloneyi* silk, freeze dried from aqueous solution. In cell with P_2O_5. (*c*) *Anaphe moloneyi* cast from trifluoracetic acid on silver chloride and rolled. The thicker specimen is not so well oriented as the thinner one; full and broken lines as in fig. 1. (*d*) Polyglycine cast from trifluoracetic acid on silver chloride and rolled.

mode is at 1558 cm^{-1}. The 1685 cm^{-1} band of polyglycine I cannot be recognized in the spectrum of polyglycine II, and this affords proof that this band does not arise from end groups or impurities.

There is a band at 1434 cm^{-1} in polyglycine I (1418 cm^{-1} in polyglycine II) which may be a CH deformation mode. It does not shift on deuteration of the NH group in polyglycine II. The expected perpendicular dichroism cannot be seen in fig. 2 but this may be a consequence of poor orientation, which affects perpendicular bands more than parallel bands. The band at 1015 cm^{-1} in polyglycine I and 1026 cm^{-1} in polyglycine II is useful for diagnostic purposes, and shows that whereas polyglycine precipitated from solution under the conditions indicated above consists wholly of form II, a mixture of I and II (with I predominating) is produced by casting from dichloracetic acid. Blout and Linsley [22] have given reasons for believing that this band is associated with a diglycyl group.

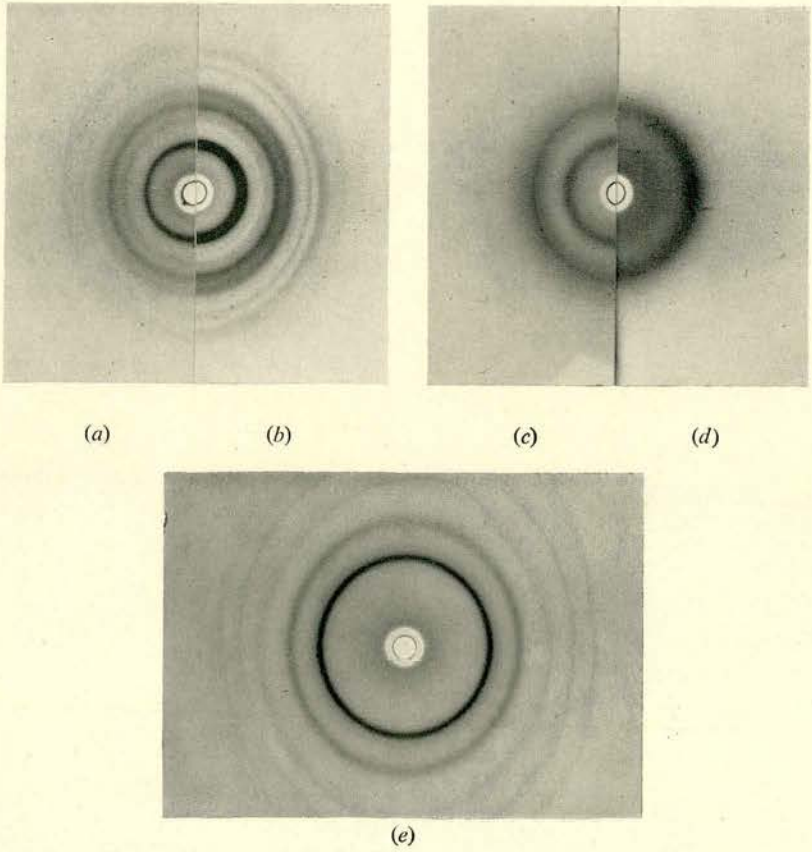

FIG. 3.—X-ray powder photographs (3-cm vacuum camera).

(*a*) Poly-L-alanine cast from dichloracetic acid. (*b*) Co-polymer L-alanine-glycine 2 : 1 cast from dichloracetic acid. (*c*) *Anaphe moloneyi* silk (cast on paraffin wax from trifluoracetic acid). The sharp ring at 4·2 Å is caused by β material produced by strain during drying. (*d*) Water-soluble *Anaphe moloneyi* silk (freeze-dried from aqueous solution). (*e*) Polyglycine II precipitated from aqueous lithium bromide by the addition of water.

[*To face page* 533.

The X-ray diffraction powder photograph of polyglycine II shows an extremely strong, sharp ring corresponding to a spacing of 4·15 Å (fig. 3*e*); this is the longest spacing which has been observed. Other, much weaker rings appear, but even on heavily exposed photographs no ring in the neighbourhood of the 1·15 Å reflection of β polyglycine is to be seen. A very small degree of orientation may be obtained by rolling polyglycine II between heated rollers; the X-ray diffraction pattern from such a specimen shows that the 4·15 Å reflection is equatorial rather than meridional.

It is certain that the 4·15 Å reflection is not from the $10\bar{1}0$ planes of an α helix arrangement, for this would lead to a crystallographic density in the neighbourhood of 3 g cm^{-3} and this is impossibly high. Since the $10\bar{1}0$ reflection always produces the strongest ring in a powder photograph of a polypeptide in the α helix form, we can conclude that polyglycine II is not in this form.

In most cases, when films of polyglycine are made by evaporation of the solvent, the extended β form is obtained, in contrast to precipitation from solution, which gives form II. However, polyglycine dissolved in formic acid containing calcium chloride may be dried by evaporation, and if the salt is then removed by extraction with ethyl alcohol, a film of polyglycine II is obtained. This film may be rolled in steam, with considerable extension, without producing any orientation of form II, but a small amount of fairly well-oriented β polyglycine is produced; double orientation of the β form may be detected. It appears, therefore, that a transition from form II to the extended chain configuration may take place.

The absence of a reflection corresponding to *ca.* 1·16 Å, which is very strong in β polypeptides, is evidence that polyglycine II has not extended, or near-extended chains. This is confirmed by examination of the optical transforms of the *c*-axis projection of the various near-extended configurations proposed by Pauling and Corey.[9, 24] From these it may be seen that in all cases a shorter spacing than 4·15 Å should appear with *greater* intensity than this ring, if the 4·15 Å spacing is assumed to correspond to the inter-plane distance along the hydrogen bonds.

The great strength of the 4·15 Å ring, together with the fact that it appears to arise from equatorial reflections, suggests the $10\bar{1}0$ reflection from hexagonally packed rods, or perhaps the coincidence of two reflections from basal planes, which would occur if the *a*- and *b*-axes were equal. It is not at present known whether polyglycine II has internal or inter-chain hydrogen bonds, and both possibilities must be considered. Of the structures with internal bonds which have been described in the literature, only the 2_7 flat ribbon or the related $2{\cdot}2_7$ helix (Donohue [4]) would give reasonable values for the density if packed in planes 4·15 Å apart. The optical transform of the $2{\cdot}2_7$ helix has been examined and from photometric measurements the values of the structure amplitudes for the chief reflections have been determined, assuming a hexagonal cell based on a value 4·15 Å for the $10\bar{1}0$ reflection. From these, the intensities of the chief rings in the powder diffraction diagram have been calculated. Although the predicted intensities agree with the observed powder photograph in one respect, namely that no orders of the 4·15 Å ring show appreciable intensity, it does not appear possible to conclude that polyglycine II consists of $2{\cdot}2_7$ helices. It is not impossible, however, that a different set of co-ordinates would lead to a predicted powder diagram more in keeping with the observed photograph. The $2{\cdot}2_7$ helix should give rise to a reflection corresponding to the residue translation 2·75 Å. However, this reflection has not a particularly large structure amplitude in polyglycine, and although its absence from an X-ray photograph of an oriented specimen would be evidence against the presence of the $2{\cdot}2_7$ helix, the fact that no corresponding ring is seen in the powder photograph is not conclusive. The multiplicity of the 00*l* reflections is much lower than that from other planes in the hexagonal system, and rings from such reflections may be weak. The possibility that polyglycine II contains 2_7 helices has not been examined, since in this case

there is no reason to expect a hexagonal structure and there are too many possibilities to make speculation profitable at this stage.*

So far, attempts to prepare poly-α-amino-*iso*butyric acid in a form corresponding to polyglycine II have not been successful, the polymer having always been obtained in a form giving the characteristic α-helix spectrum and X-ray powder diagram. This suggests that the absence of an asymmetric carbon atom is not the primary cause of polyglycine not folding in the α-helix configuration and no trace of any α-helix structure has been obtained in polyglycine prepared by any of the methods described.

SILK FIBROIN

Turning now to the spectra of β silk in fig. 1*c* and 2*c*, the great similarity of *Bombyx mori* and of *Anaphe moloneyi* fibroin in the region 800-1500 cm^{-1} may be noted. The great similarity between the spectra of silks and of β-poly-L-alanine in the region of overtone and combination bands has already been commented on.[25] According to Lucas, Shaw and Smith,[17] *Anaphe moloneyi* silk contains approximately 39 glycine+50 alanine out of a total of 100 residues, whereas *Bombyx mori* silk has 41 glycine and 28 alanine, with a correspondingly higher proportion of bulky side chains than is found in Anaphe silk. The similarity between the two fibroin spectra shows how dominant is the effect of the glycine and alanine residues, an effect which has been pointed out by Astbury, Dalgleish, Darmon and Sutherland[19] on the basis of frequency measurements on an unspecified silk. The correspondence shown in fig. 1*c* and 2*c* is greatly enhanced by the similarity of the dichroic character of the bands.

The similarities in the frequencies and dichroism of the amide bands in the two silks may be seen by comparing fig. 2*c* with fig. 1 in an earlier publication.[21] The weak but markedly dichroic (parallel) band at 1697 cm^{-1} in both materials must have the same origin as the band at 1685 cm^{-1} in polyglycine I referred to above. It is perhaps a combination band; the high dichroism may mean that it is associated with the crystal lattice, but this is not necessarily the case, since with β structures which are produced by stretching a folded chain the whole structure is usually well oriented.

Amide-group bands found in poly-L-alanine have been discussed elsewhere.[23] In fibroin, the strong parallel band at 1231-1235 cm^{-1} is almost certainly such a band, and may contain contributions both from glycine and from alanine residues (see table 1). The parallel band at 1266 cm^{-1} is not found in polyglycine or in poly-L-alanine.

TABLE 1.—WAVE NUMBERS OF CORRESPONDING ABSORPTION BANDS IN SPECTRA OF FIBROIN, POLYGLYCINE AND β-POLY-L-ALANINE (cm^{-1})

Bombyx mori	928	978	999	1166	1235	1447	1453
Anaphe moloneyi		975	1001	1168	1231	1446	1452
polyglycine I (β)			1015		1235		
poly-L-alanine (β)	924	966		1168	1220	1446	1454
dichroic character	⊥	∥	∥	∥	∥	⊥	∥
origin				CH_3	amide	CH_3	CH_3

As shown in table 1, a number of the stronger bands may be correlated with the glycine and the alanine residues of fibroin. The assignment of three of these bands to methyl group frequencies is reasonably certain. The splitting of the 1450 cm^{-1} asymmetrical CH_3 deformation mode has been ascribed to steric effects.[23] The 924 cm^{-1} and 966 cm^{-1} bands are evidently skeletal modes, since they appear in β-poly-L-alanine but are not present in the spectrum of the α form.

* *Note added in proof.*—Following a brief note describing the occurrence of polyglycine II,[27] a structure for this material, consistent with our observations, has been proposed by Crick and Rich.[28]

WATER-SOLUBLE SILK

The water-soluble form of Bombyx or Anaphe silk, prepared by any of the methods described above and in the literature, is not in the typical β configuration. This is shown by the frequency of the C=O band (1657-1660 cm^{-1}), by the absence of a β-type X-ray diffraction diagram, and also by the fact that a small amount of extended crystalline (β) material is sufficient to make the whole insoluble. However, the spectrum of water-soluble Anaphe silk varies with the mode of preparation, and is markedly different from that of Bombyx silk made water-soluble by the agency of lithium bromide and cast from water (fig. 1*d*, fig. 2*a* and 2*b*). This is in marked contrast to the similarity of Bombyx and Anaphe silk spectra in the β form.

It would be expected that *Anaphe moloneyi* silk, which is to a great extent a co-polymer of glycine and alanine, would readily form an α helix, and this material when cast from trifluoracetic acid does give an X-ray diffraction photograph with a prominent ring corresponding to a spacing of 7 Å. Poly-L-alanine in the α-helix form has a corresponding $10\bar{1}0$ spacing of 7·40 Å;[26] a co-polymer of L-alanine and glycine (2 : 1) has a reflection at 7·37 Å (fig. 3*b*), and no doubt the shorter spacing in silk is a consequence of the presence of the smaller glycine residues. The infra-red spectrum, too, shows several bands which are common to both α-poly-L-alanine[23] and *A. moloneyi* silk cast from trifluoracetic acid (fig. 2*a*). Omitting those bands which are common to both α and β forms of poly-L-alanine, and which are probably caused by methyl group modes, the bands at 1274, 1308 and 1331 cm^{-1} in α-poly-L-alanine may be compared with those at 1270, 1298 and 1331 cm^{-1} in *A. moloneyi* silk. We have therefore good grounds for believing that α helices are present in *A. moloneyi* silk when cast from trifluoracetic acid. However, comparison of the X-ray diffraction photograph of this material with that of the alanine-glycine co-polymer suggests that perhaps some other structure besides that of the α helix is present.

Turning now to the biologically more interesting Anaphe silk prepared by freeze-drying an aqueous solution, it is striking that the bands characteristic of the α helix form of poly-L-alanine are weaker, relative to other bands, in fig. 2*b* than in 2*a*. The weakening is especially marked in the case of the 1270 and 1298 cm^{-1} bands. An additional, and perhaps important difference between these spectra is that the band characteristic of glycine residues is at 1005 cm^{-1} in fig. 2*a* and at 1013 cm^{-1} in fig. 2*b*. In the β form of *A. moloneyi* silk this band is at 1001 cm^{-1}. It may perhaps be significant that the wave-number difference for this band between polyglycine I and II is 11 cm^{-1} and the corresponding figure for Anaphe silk (β form and freeze-dried form) is 12 cm^{-1}.

A further difference between the spectra in fig. 2*a* and 2*b* is in the considerably greater breadth of the C=O and NH deformation bands in the latter. The important question which arises from the observations just described is whether the differences between the two methods of preparing water-soluble Anaphe silk arise only from differences of crystallinity, or whether some differences in molecular configuration occur. This question cannot at present be answered by infra-red spectroscopy (in the absence of a method of orienting the water-soluble form). X-ray powder photographs do, however, reveal a striking difference between the use of trifluoracetic acid and water as solvent (see fig. 3). The characteristic feature of an α helix powder photograph, the strong $10\bar{1}0$ ring (the inner ring in the specimen prepared from trifluoracetic acid) is absent from the diagram when the specimen is cast from water and freeze-dried. In the latter case, practically the whole of the scattered radiation is concentrated in a ring centred on a spacing of *ca.* 4·35 Å. The powder diagram of a poorly crystalline assembly of α helices should retain the main intensity distribution of the well-crystallized powder and in particular any region which in the crystalline powder diagram is strong should remain appreciable when the molecular arrangement is more random, even though the whole pattern becomes more diffuse. This is shown by the fact that the

X-ray diffraction patterns of freeze dried poly-DL-alanine (from aqueous solution) and of freeze-dried poly-γ-benzyl-L-glutamate (from benzene) show strong, though broad rings characteristic of the α helix. It seems, therefore, on the basis of the evidence available reasonable to conclude that *A. moloneyi* silk freeze-dried from aqueous solution does not contain α helices.

The infra-red spectrum of freeze-dried *A. monoleyi* suggests the absence of a β configuration, but the single, rather broad peak at 1660 cm^{-1} need not necessarily mean that the molecules are in a *regular* folded form. It does, however, suggest that the environment of all the C=O groups is substantially the same. A perfectly regular fold appears to be excluded by the single, broad ring shown by the X-ray diffraction diagram. This would appear to leave two possibilities.

(i) The hydrogen bonds may all be formed within one chain in a fold which allows considerable molecular flexibility by rotation around the α carbon single bonds.

(ii) The hydrogen bonds are random, comprising both inter- and intra-chain, but nevertheless the immediate environment of the C=O groups does not vary greatly throughout the material.

The spectrum of water-soluble Bombyx silk shows even less evidence of characteristic α-poly-L-alanine bands than does *Anaphe moloneyi* silk freeze-dried from aqueous solution. The characteristic polyglycine band appears at 1015 cm^{-1}, which is 16 cm^{-1} higher than in the β form. Even when water-soluble Bombyx silk is prepared in the form of quite clear films, a considerable amount of continuous absorption is present (fig. 1*d*). This may perhaps be a consequence of the diversity of side chains in Bombyx silk.

The X-ray powder photograph of Bombyx silk cast from aqueous solution gives a broad ring, with limits corresponding to 3·6 Å and 5·7 Å and probably has a similar origin to that produced by freeze-dried Anaphe silk.

We are indebted to Dr. Bamford, Mr. W. E. Hanby and Dr. D. G. H. Ballard for the polyglycine, to Mr. Stroud for lithium bromide silk, to Dr. Smith of the Shirley Institute for *Anaphe moloneyi* silk and to Mr. L. Brown of Courtaulds X-ray laboratory for the X-ray photographs.

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Structure of Polyglycine

NEARLY twenty years ago, Meyer and Go[1] described the X-ray diffraction powder photograph of some glycine peptides, and found that different photographs were obtained from polyglycine according to the method of preparation. When the polymer was made by decomposition of the N-carboxy glycine anhydride in the presence of pyridine, reflexions corresponding to 4·4 A. and 3·45 A. were observed; but the same polymer precipitated by water from aqueous lithium bromide solution gave a photograph in which a reflexion at 4·15 A. was predominant. Subsequent observations[2–4] have related to the first form, which may be produced by casting from solution in dichloracetic acid or in trifluoracetic acid. It will be convenient to refer to this form as polyglycine I, and to the form characterized by the 4·15-A. reflexion as polyglycine II.

It has recently been found[5] that the two forms have different infra-red spectra. Whereas polyglycine I is a typical β-polypeptide, with absorption bands at 1,630 cm.$^{-1}$ (C=O) and 1,530 cm.$^{-1}$, polyglycine II has bands at 1,648 cm.$^{-1}$ (C=O) and 1,558 cm.$^{-1}$. The C=O mode of the second form, therefore, does not fall within the range found for either α- or β-synthetic polypeptides[6]. Other bands enable polyglycine I and II to be distinguished; the most convenient are at, respectively, 1,015 cm.$^{-1}$ and 1,026 cm.$^{-1}$. Under suitable conditions of precipitation, which were found by examining the infra-red spectra, polyglycine II can be obtained in a pure form. The X-ray diffraction diagram of this pure form is shown in Fig. 1. It would appear that Meyer and Go's polyglycine (shown in Plate II of their paper) was a mixture of I and II.

The diffraction pattern of polyglycine I contains a reflexion at a spacing of 1·16 A. A reflexion occurring at this spacing in other β-polypeptides and silks is the 006 reflexion (*c* is the molecular chain axis), and if polyglycine consists of extended polypeptide chains, strong reflexion would be expected very near this spacing whether the chains were packed in a rectangular or a triclinic cell[4]. We have found no reflexion corresponding to a spacing in the neighbourhood of 1·16 A. in polyglycine II, showing that the two forms do not arise from different ways of packing extended (or nearly extended) chains; the chains in the two forms must have different configurations. This also follows from consideration of

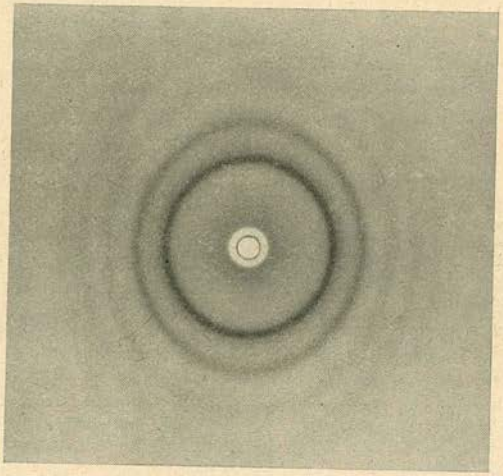

Fig. 1

the relative intensities of the rings in the two powder photographs. The relative intensities of the equatorial reflexions from a given crystalline arrangement of extended (β) chains may be estimated from optical transforms of the *c*-axis projection. It is readily seen that the 3·45-A. reflexion should be somewhat stronger than the 4·4-A. reflexion if, following Astbury[8], these are indexed as 010 and 100 respectively; and in fact this is observed in polyglycine I, the β-form. However, to interpret polyglycine II in terms of a nearly extended chain would require us to take the very intense 4·15-A. reflexion as 100, with the next strongest (3·1 A.) as 010, in order to get a reasonable molecular packing. The predicted intensities for such an arrangement are quite different from those observed.

The very intense 4·15-A. reflexion appears to come from planes which are parallel or nearly parallel to the fibre axis (see below). The high intensity shows that the electron density has a very marked periodicity of 4·15 A., and it is reasonable to conclude that this is the separation of layers of polypeptide chains, possibly in hexagonal array. The α-helix (which would give hexagonal packing) can be excluded, since with such close approach of chains the density would be impossibly high (more than 3 gm./c.c.).

Of the various structures which have been proposed for polypeptide chains, only two appear to provide reasonable values for the density, and optical transforms of these, the $2 \cdot 2_7$ helix[7] and the unrotated parallel polar sheet[8], have been examined. Space-

filling models of the polar sheet show that interplanar distances of about 4·15 A. and 3·6 A. occur; but the optical transform shows that these reflexions should appear with comparable intensities, whereas the X-ray photograph shows a very weak ring corresponding to 3·8 A. and an overwhelmingly strong 4·15-A. reflexion (see Fig. 1). It seems, therefore, unlikely that a polar sheet structure is present.

Transforms of the $2\cdot2_7$ helix based on a $10\bar{1}0$ reflexion of 4·15 A. lead to a prediction of intensities in general agreement with those observed in the X-ray powder photograph; but the details are not in agreement. In particular, no reflexion corresponding to the residue repeat 2·75 A. is seen. In a triclinic cell, such a reflexion would not be expected, since there is no reciprocal lattice point corresponding to 2·75 A.; but clearly such a possibility cannot be examined profitably until a fibre photograph is obtained. The density predicted for such an arrangement of $2\cdot2_7$ helices is 1·67 gm./c.c., which is considerably higher than the measured density of polyglycine II (1·43 gm./c.c.).

The methods by which polyglycine II may be prepared suggest that it might be an intra-chain hydrogen-bonded structure. Casting from any solvent such as dichloracetic acid invariably produces a predominance of the β-form, and we have found that precipitation from solution is generally necessary in order to prepare form II. The only method we have found which produces coherent films of form II is to prepare films in the presence of a salt. The polymer is dissolved in a mixture of formic acid, and calcium chloride and the formic acid dried off. The calcium chloride may then be washed out with alcohol, leaving a spongy film. It appears likely that the effect of the salt is to reduce the probability of intermolecular hydrogen bonding. If films made in this way are rolled, a small amount of orientation of form II is obtained which indicates that the 4·15-A. reflexion is probably equatorial rather than meridional. In addition, if the films, which from infra-red and X-ray examination are found to contain no β-material, are rolled hot, a small amount of doubly oriented β-material is produced. The β-material is quite well oriented, the mean direction of the chain axis lying in the direction of rolling; a similar orientation is observed in the $\alpha \rightarrow \beta$ transformation in other synthetic polypeptides.

These facts suggest very strongly that the molecules of form II are folded and that on rolling a number are pulled out into the β-form. It would appear that here we have strong evidence for a configuration of the polypeptide chain which is outside the range of structures hitherto accepted.

No evidence of the α-helical form of polyglycine has been found, except observation of parallel dichroism of the NH-band in a specimen which had been stretched in polystyrene[6]. Recent observations suggest that this may really be a 'cross β' structure, since the C=O band accompanying the dichroic NH band has the β- rather than the α-frequency.

The fact that the 1,015 cm.$^{-1}$ band of polyglycine disappears when the β-form is converted into form II shows that the absence of this band in the spectrum of polymers containing glycine is no proof that the structure is one in which there are no glycyl–glycyl bonds. Especially in the case of alanine–glycine copolymers, and in silks, the possibility that frequency changes are caused by change of molecular configuration must be considered. Since criteria for the recognition of α- and β-forms have now been known for some years[6], we consider that these possibilities should be considered before conclusions on the chemical constitution of silk are based on the appearance or absence of bands in the spectrum[9]. The work we have here reported shows that there exists, in polyglycine at least, a configuration of the polypeptide chain other than the α-helix and the normal β-forms.

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CONFIGURATIONS OF SILK FIBROINS AND SYNTHETIC POLYPEPTIDES

by

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INTRODUCTION

Some years ago it was reported from this laboratory that the infra-red spectra of water-soluble silk and the same silk rendered insoluble by various means were different[1]. The frequency of the carbonyl band of silk rendered soluble by, for instance, solution in aqueous lithium bromide followed by dialysis is at 1660 cm^{-1}, whereas the same band has its maximum at 1630 cm^{-1} in lithium bromide silk which has been rendered insoluble by precipitation with ethyl alcohol. In silk gut this band is also at 1630 cm^{-1} [2]. The wave numbers of the carbonyl band were so close to those found in α and β synthetic polypeptides that the suggestion was made that water-soluble silk was folded in the same way as an α synthetic polypeptide and that the spinning of silk was accompanied by unfolding of these chains into the extended β-form. This was suggested not only by the evidence of infra-red spectra, but also because it had been shown that synthetic polypeptides in the α-form were more soluble than those in the β-configuration[3]. The difference in solubility is a consequence of the fact that in α synthetic polypeptides the CO $\cdots$ HN hydrogen bonds are formed within one chain whereas in the β-configuration these bonds form a link between neighbouring chains[3,4].

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At the time when the first observations on the spectrum of water-soluble silk were made, the α-helix had not been proposed for α-polypeptides. Since this structure was described by PAULING, COREY AND BRANSON[5] a remarkable amount of evidence has accumulated which shows the essential correctness of the α-helix in simple polypeptides. This, however, has not led to a convincing demonstration that α-helices are the main structural unit in globular proteins. In fibrous proteins, however, the keratins probably contain such helices wound into super-helices[6,7] and we have recently[8] found evidence that the fibroin of *Anaphe moloneyi* cocoons may be obtained in a water-soluble form which gives an X-ray diffraction pattern resembling a powder photograph of α-poly-L-alanine. This pattern, as well as infra-red spectra, provides good evidence for the occurrence of α-helices in a film obtained by drying a solution of *Anaphe* fibroin in trifluoroacetic acid. When, however, the fibroin was dispersed in water, the natural medium of proteins, no evidence of the presence of α-helices in the dried film could be found, although the configuration in this case was certainly not the extended, crystalline β-form.

With films of regenerated *Bombyx mori* fibroin prepared from aqueous media, the results depend on the conditions. Casting at 100° C produces films whose X-ray diffraction pattern is completely amorphous[1]. When the native gel from the silk gland is dried at room temperature the X-ray pattern may show a ring corresponding to 7.5 A accompanied by other rings[9]. The 7.5 A ring is probably the $10\bar{1}0$ reflection of a hexagonal arrangement of α-helices (compare poly-L-alanine in which the corresponding value is 7.4 A). However, this ring is by no means dominant, as it always is in the pattern obtained with simple α-polypeptides, and it is certain that in films of *Bombyx* silk the α-helix configuration, when it occurs, only accounts for part of the structure.

It appeared likely that the problem of the nature of water-soluble silk fibroin could usefully be studied by examining the properties of a number of different kinds of silk, and by comparing them with those of synthetic polypeptides containing alanine and glycine. We have accordingly collected information on the infra-red spectra of films of these substances, prepared under different conditions, and have drawn some tentative conclusions. The number of solvents from which silk films may be cast is very small, and we have confined our choice to water, trifluoroacetic acid and dichloroacetic acid. Not all natural silk fibres are soluble in the last two materials, but it has been found that if a water-soluble preparation is first made by methods indicated in the next section, this form may be dissolved in the other solvents.

EXPERIMENTAL

Water soluble forms of the materials used have been prepared as follows:

Bombyx mori fibroin. Solution in aqueous lithium bromide, followed by dialysis against water. Films are cast on a mercury surface at 100° C.

Antherea mylitta fibroin (tussah). Solution in saturated aqueous lithium thiocyanate, followed by dialysis against water. The resulting solution is freeze-dried. Parts of the cocoon do not disperse in the salt solution.

Anaphe moloneyi fibroin, and L-alanine–glycine 1:1 co-polymer. Solution in the minimum quantity of trifluoroacetic acid, to which is added water to make a 1 % solution, which is freeze-dried.

Anaphe infracta fibroin. Solution (1 % wt/volume) in trifluoroacetic acid, from which a film is cast on mercury at room temperature in a dry atmosphere. This method may also be used for *Anaphe moloneyi*.

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Films which were not cast on mercury were cast on plates of thallium bromo-iodide. Solutions of silk in trifluoroacetic acid must be used as quickly as possible, since degradation occurs in this solvent. The infra-red spectra were recorded with a Grubb-Parsons spectrometer fitted with a rock salt prism, and are reproduced in Figs. 1 and 2.

DISCUSSION

Fig. 1 shows that the carbonyl band of the silks and alanine-glycine polymers has peaks at about 1660 and 1630 cm^{-1} whose relative heights depend on the solvent used as well as on the polymer. In simple polypeptides these peaks are characteristic of α-helix and β-forms[4].

In proteins, however, the 1660 cm^{-1} band is certainly not always associated with the α-helix form. For instance, in collagen, where there are no α-helices, the carbonyl band has this wave-number. We shall refer to the configuration associated with the 1660 cm^{-1} band simply as folded, and will consider the nature of the fold at the end of this section.

In the films cast from dichloroacetic acid the tendency is for the height of the β peak to increase with an increasing proportion of glycine residues. Since all simple polypeptides of high molecular weight hitherto examined appear to be in a folded form when cast from this solvent, with the single exception of polyglycine, this is to be expected. However, the proportion of glycine to other residues in *Antherea mylitta* fibroin is not much lower than in some other polypeptides examined, and the very small proportion of β-chains indicated by the spectrum is surprising.

When trifluoroacetic acid is used as solvent, all the materials except polyglycine are obtained in a folded form, and the 1630 cm^{-1} band is absent.

Casting the silks and the L-alanine-glycine 1:1 co-polymer from aqueous solution give results which depend rather markedly on conditions, and which are therefore difficult to reproduce. The tendency for a β peak to appear in the carbonyl band in films cast from water is most marked when the polymer contains few residues other than alanine and glycine.

Of the silks and co-polymers examined, the fibroin of *Antherea mylitta* shows the greatest intensity of the 1660 cm^{-1} peak under all

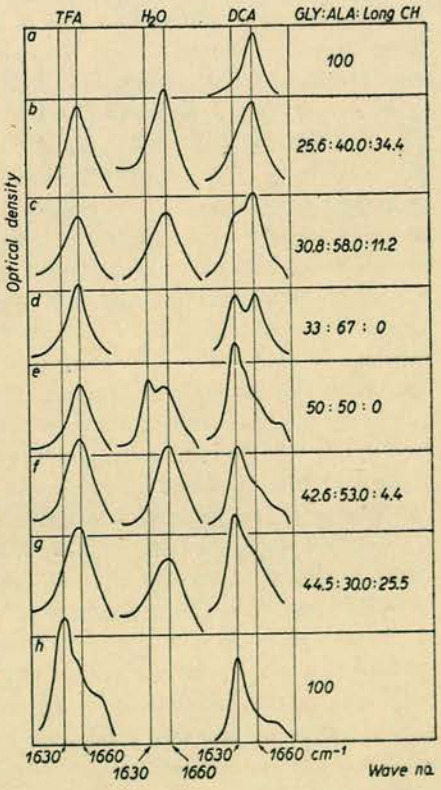

Fig. 1. The carbonyl band in the infra-red spectrum of silks and synthetic polypeptides cast from trifluoroacetic acid, from water and from dichloroacetic acid. *a*, Poly-L-alanine; *b*, *Anthera mylitta* fibroin; *c*, *Anaphe infracta* fibroin; *d*, copolymer L-alanine: glycine 2:1; *e*, copolymer L-alanine:glycine 1:1; *f*, *Anaphe moloneyi* fibroin; *g*, *Bombyx mori* fibroin; *h* polyglycine.

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conditions. The spectrum of this material in the region 800–1400 cm^{-1} (cast from water at room temperature) is shown in Fig. 2, along with that of α poly-L-alanine. The resemblance is striking, and every band in the spectrum of the synthetic material may be identified in that of the silk.

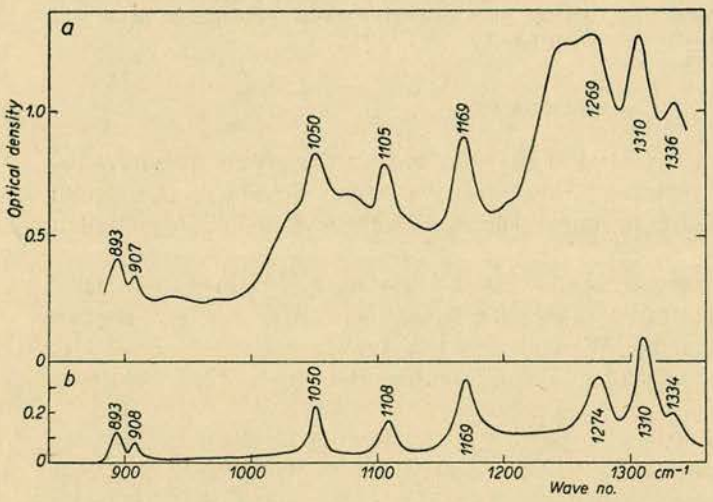

Fig. 2. Infra-red spectra. *a*, *Antherea mylitta* (tussah) fibroin cast from water. *b*, Poly-L-alanine (α-form cast from dichloroacetic acid).

It is known that a number of these bands are characteristic of α poly-L-alanine, and do not appear in the β-form. The band at 908 cm^{-1} is particularly important, for it appears to be associated with crystalline regions[10]. It may be concluded that in films of *Antherea mylitta* fibroin cast from aqueous solution a large part of the alanine residues are in α-helices, and this is confirmed by the X-ray powder photograph of this material, which has a dominant ring corresponding to a spacing of 7.4 A, identical with the $10\bar{1}0$ reflection obtained from an unoriented powder photograph of a rather poorly crystalline specimen of poly-L-alanine.

The discovery of α-helix configurations in a film of water-soluble silk is interesting, but it is remarkable that both infra-red and X-ray diffraction techniques reveal the presence of α-helices of poly-alanine, and that the considerable proportion of larger residues seems to contribute nothing to the magnitude of the $10\bar{1}0$ spacing. These results recall an earlier observation that the crystalline regions of β poly-L-alanine and *Antherea mylitta* (tussah) silk are isomorphous[11,12] and their X-ray diffraction patterns are almost identical. A possible deduction is that many of the alanine residues in this silk are concentrated in a particular region of the chain, and that only such regions form crystallites. If this were the case, the alanine residues would impose much of the character of poly-L-alanine on the material. Some such explanation appears to be demanded when results obtained with *Antherea mylitta* fibroin are compared with those we have found with the two *Anaphe* fibroins. Although some of the bands characteristic of α poly-L-alanine were recognized in the spectrum of *Anaphe moloneyi* cast from trifluoroacetic acid[8], we were unable to detect the 908 cm^{-1} band, and have again failed to find it in the spectrum of *Anaphe infracta*, although in both these silks the proportion of alanine residues is considerably greater than in *Antherea mylitta* fibroin. In the diffraction pattern of *Anaphe moloneyi*, the $10\bar{1}0$ spacing is 7.1 A, appreciably less than in poly-L-alanine, and this suggests that there is glycine as well as alanine in the crystalline regions. This is further borne out by the presence of the 001 reflection corresponding to the fibre axis pseudo-repeat 6.94 A in the diffraction pattern of natural (β) *Anaphe moloneyi* silk, which shows

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that the chains in the crystalline regions do not have a 2-fold screw axis (as in poly-L-alanine) associated with the pseudo-repeat along the chain.

The observation that, in general, the films cast from dichloroacetic acid have a β-component, unlike those cast from trifluoroacetic acid, is interesting. Another striking difference is observed when water is added to silk solutions in these two solvents. All the polypeptides *a–h* (Fig. 1) are immediately precipitated from solution in dichloroacetic acid. With solutions in trifluoroacetic acid, however, no precipitation occurs on adding excess water except to poly-L-alanine, polyglycine and the 2:1 alanine-glycine co-polymer. The resulting dispersion is not a stable solution; a gel is formed in the course of a few hours or days. These observations are compatible with a random-coiled form of the polymer with the peptide groups mainly hydrogen bonded to the dichloroacetic acid molecules; in solution in trifluoroacetic acid, however, the α-helix may be the more stable form. The difference between the two solvents is perhaps connected with the CH group in dichloroacetic acid, which will have appreciable hydrogen-bonding capacity.

The dispersions of silks in water are unstable and films cast from a dispersion of the same silk under apparently identical conditions do not always yield identical spectra. Temperature is an important factor, and the two *Anaphe* silks (which are particularly unstable) contain larger proportions of β-polypeptide when cast at room temperature than when cast at 0° C. Although all four silks examined and the alanine-glycine 1:1 co-polymer can be cast as films from water without any appreciable β peak, only *Antherea mylitta* gives films whose molecular chains are demonstrably in the α-helix form. The presence of α-helices in the solid film is not evidence of such structures in the solution from which the films are cast. However, it appears likely that molecules which are in the α-helix form in solution would to a great extent preserve their configuration when the solution is evaporated.

KRATKY[13] has examined the small-angle scattering of X-rays from native gels of *Bombyx mori* fibroin, and has interpreted his results as showing that the silk is in the form of α-helices. The corrections applied to the observations are considerable, however, since what is initially observed is the combined scattering of fibroin and water, and we consider that confirmation of the result is desirable. In the experiments which we have described, the silk dispersion is not native, but regenerated, and the initial concentrations employed in casting films are much lower than in native silk gel. If the fibroin in our aqueous dispersions were in the form of α-helices, it is difficult to see why we should not always find this form in the dried film, provided that conditions are such that no β material is formed. It appears likely that, as SCHELLMAN[14] has suggested, the free energy of stabilization of an α-helix in aqueous solution is of the order kT when the effects of side chains are neglected. We may accordingly expect a comparatively narrow range of conditions over which the transition from an α-helix to a random coil of solvated chains takes place. However, it is clear that SCHELLMAN'S theory is not strictly applicable to silk, for the process is not reversible—the random coil may produce intermolecular links of the β-type, and this process when it goes far enough leads to insolubility of the polymer. We suggest that when conditions are such that the α-helix becomes unstable, a random arrangement of 13-, 10- and 7-membered intra-chain hydrogen bonded rings may be formed within each polymer chain. This is the reverse process of that suggested by CRICK[15], who pointed out that if a polypeptide chain assumed, for instance, the 2.2_7 configuration (with 7-membered

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rings) under conditions when minimum potential energy was the chief consideration, the chain would go over into the more stable α-helix form by opening the rings and reforming as 13-membered rings. It is also possible that in addition to the intra-chain bonds in silk, some random inter-chain bonding may occur and peptide groups may be hydrogen-bonded to water. It seems that, whereas the pleated sheet type of inter-chain hydrogen bonding gives a structure in which the carbonyl mode has a wave-number 1630 cm^{-1}, in other kinds of inter-chain bonding this wave-number is considerably higher. For instance, in polyglycine II the carbonyl band occurs at 1648 cm^{-1} [8]; the structure appears to have inter-chain hydrogen bonds which form a hexagonal net[16]. If the recent structure proposed for collagen is basically correct[17] there are many inter-chain hydrogen bonds, and as mentioned above the carbonyl band has a wave-number 1660 cm^{-1}.

For reasons which are not yet apparent, silk fibroin in aqueous dispersion forms a system in which equilibrium is not quickly reached. This property is essential to the process of silk spinning. The function of the glycine residues in silk is to produce a polypeptide whose most stable form is the β-configuration, and it would appear that the necessary conditions can be reached with considerable variations in the glycine content. It is evident that much remains to be done before silk spinning is understood, though some of the mechanism begins to be apparent.

The results of work on X-ray diffraction briefly reported in this paper form part of an investigation which we are carrying out in collaboration with Mr. L. BROWN and Miss E. M. CANT. We are indebted to Mr. W. E. HANBY and Dr. BALLARD for the synthetic polypeptides which we have used.

SUMMARY

The infra-red spectra of a number of different kinds of silk are compared with spectra of synthetic polypeptides prepared from L-alanine and glycine. It is shown that the molecular configuration depends on the composition of the material and on the solvent used for the preparation of the specimen. In the case of films of water-soluble *Antherea mylitta* (tussah) silk, there is strong evidence for α-helices consisting mainly of L-alanine residues. These results are discussed in relation to the configuration of the molecules in solution.

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OPTICAL ROTATION AND INFRA-RED SPECTRA OF SOME POLYPEPTIDE AND PROTEIN FILMS

OPTICAL ROTATION AND INFRA-RED SPECTRA OF SOME POLYPEPTIDE AND PROTEIN FILMS

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Refinements of technique have enabled the dispersion of the optical rotation of solid films to be measured in the visible and near ultra-violet regions. Such measurements have been made for a series of polyalanines containing different proportions of D- and L-residues, for the sodium and potassium salts of poly-L-glutamic acid and also for some protein films. The infra-red spectra of these films have also been observed. The polyalanine films show the characteristic dispersion of the α-helix, but the other materials do not. Since all these films have carbonyl absorption bands at *ca.* 1660 cm^{-1}, it is evident that this frequency is associated with two or more configurations of the polypeptide chain. With *Bombyx* silk films cast from aqueous solution, a random coil appears possible.

Infra-red spectra and X-ray diffraction patterns of polypeptides and fibrous proteins have chiefly been made on solid films, because the technique and interpretation is often more difficult if the material is in solution. On the other hand, measurements of optical rotation, which have recently yielded important information in connection with the α-helical configuration of polypeptides (Doty and Yang,[1] Moffitt and Yang,[2] Elliott, Hanby and Malcolm,[3] Yang and Doty,[4] Moffitt, Fitts and Kirkwood,[5] Downie, Elliott, Hanby and Malcolm [6]) are much more easily made on liquids and recent published work has been restricted to solutions. Since the origins of the frequencies which characterize different polypeptide configurations have never received a satisfactory explanation and are empirical observations, it has seemed desirable to fill certain gaps in our knowledge by measuring the optical rotations of solid films on which observations of infra-red spectra and of X-ray diffraction patterns could be made. This has clarified a somewhat anomalous and unsatisfactory situation concerning the configuration of *Bombyx* silk cast from an aqueous dispersion.

EXPERIMENTAL

METHODS FOR MEASURING OPTICAL ROTATION OF FILMS

The peculiar difficulties which arise in measuring the optical rotation of films (as distinct from liquids) are caused by birefringence from strain, from local orientation of the polymer molecules or from birefringent foreign bodies in the film. Birefringence in the specimen produces an error in the setting of the analyzing polarizer and causes the field to be more or less brightly illuminated. This diminishes the sensitivity of a visual or a photo-electric polarimeter, since source fluctuations become troublesome when the field is bright. To prepare specimens which are sufficiently homogeneous, it is usually necessary to make a number of thin films rather than a few thick ones.

In practice, reasonably accurate measurements can only be made with a suitable photo-electric polarimeter, and we have used one of our own design (Malcolm and Elliott [7]). Since a small amount of specimen birefringence is usually present, it is desirable to rotate the specimens round the axis of the polarimeter tube in which they are housed. Initially this was done in steps by hand, and the average of a number of such readings was taken. Later a small motor was fitted to rotate the specimens at a high speed, and only one reading was then required. Careful filtering and drying in filtered air is needed to

produce clean films. When the number of films required is large (say twenty or more), or when the film thickness is irregular, it is advantageous to immerse the films in a suitable liquid to reduce reflection and distortion of the polarized beam. Edwards' silicone fluid 703 as used for diffusion pumps is suitable for some purposes. Its disadvantage lies in the presence of dissolved air, some of which may form a bubble which, owing to centrifugal forces, remains on the axis of rotation just in the middle of the field of view. Frequent evacuation of the filled tube is needed to remove this air. Styrene is also suitable in some cases.

Measurements of the dispersion of optical rotation were made initially with filtered light from a mercury lamp. Later a small monochromator was substituted for the filter, since it is difficult to get sufficient spectral purity and intensity with filters.

The films were cast on small squares of thin sheet glass which were annealed after cutting. The weight per unit area was obtained by marking a small central square with a razor blade and subsequently weighing the film contained within this square. Since the total amount of this material was usually only a few milligrams, the principal source of error lies in this determination. It is not necessary to know the specific gravity of the polymer in order to calculate the specific rotation, for the latter quantity may be defined as the rotation produced by 100 g of material in a column of 1 sq. cm cross-section.

The accuracy of measurement of optical rotation in films naturally falls short of that which can be obtained with solutions, for with the maximum number of films which can in practice be used, the rotation produced is small. In the work to be described, the angle of rotation varied from 0·04° to about 1·2°; the polarimeter could be set to about 0·001°.

RESULTS

POLYALANINE

Four polyalanines, copolymers containing different ratios of L- and D-alanine were examined as films. They were cast at room temperature from 5 % solutions in dichloracetic acid into an alcohol. To produce the best films, it was found desirable to use *iso*butyl alcohol for the D-polypeptide, *n*-propyl alcohol for the next two members of the series and methyl alcohol for the last one. D-residues were present in excess in these polymers, but to facilitate comparison with earlier results [3, 6] they are recorded and plotted as if the L-component had been predominant. The films were well washed in ethyl or methyl alcohol and air-dried. Films of suitable thickness were cast on plates of thallium bromo-iodide under the same conditions to provide specimens for infra-red examination.

Residue rotations,[6] corrected for an assumed refractive index of the polymer of 1·5 are shown in fig. 1 plotted against the fraction of L-residues for five different wavelengths. It will be seen that all the observed rotations for polyalanine films are negative, and by comparison with rotations observed for this polymer in solution it might be thought that the polyalanine films were in some form other than an α-helix. This, however, is not the case, for the dispersion is of the "anomalous" type associated with α-helices. As shown by Moffitt and Yang [2] the dispersion of the optical rotation produced by α-helices is of the form

$$[R_{\mathrm{vac}}]_\lambda = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2},$$

and on plotting $[R_{\mathrm{vac}}](\lambda^2 - \lambda_0^2)$ against $1/(\lambda^2 - \lambda_0^2)$ a straight line of slope $b_0\lambda_0^4$ is obtained. The results here presented do not allow an independent determination of λ_0, but when this constant is assumed to have the value 2120 Å, as found by Moffitt and Yang for poly-γ-benzyl-L-glutamate, the curves shown in fig. 2 are obtained. For polyalanine films whose L/(D + L) composition is 1·0, 0·9 and 0·8 the values of b_0 are respectively − 475, − 500 and − 505 deg. cm² per decimole. These may be compared with − 630° for poly-γ-benzyl-L-glutamate [2] and − 460° for polyleucine with L/(D + L) equal [6] to 0·875.

When the optical rotation contains a contribution from an arrangement of α-helices, the plot of $[R_{\mathrm{vac}}]$ against L/(D + L) gives a linear part of greater or less length which on extrapolation does not go through the origin.[3, 6] This is clearly the situation in fig. 1. In this graph, for reasons discussed below, the linear relationship appears to be restricted to points for which L/(D + L) is 0·8 or more. Over this range, the linearity of the plot and the approximate constancy of b_0 both show that one sense of helix is predominant. The plots extrapolate to intersect the $[R_{\mathrm{vac}}]$ axis at points which represent the values of this quantity for a right-handed helix of *meso* composition. Although such extrapolation

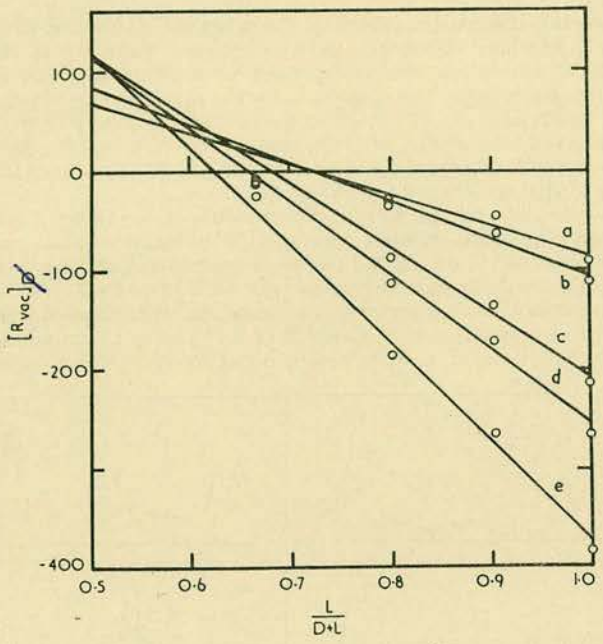

FIG. 1.—Optical rotations of solid films of polyalanine plotted against composition, for different wavelengths: *a*, 5780 Å; *b*, 5461 Å; *c*, 4358 Å; *d*, 4047 Å; *e*, 3663 Å.

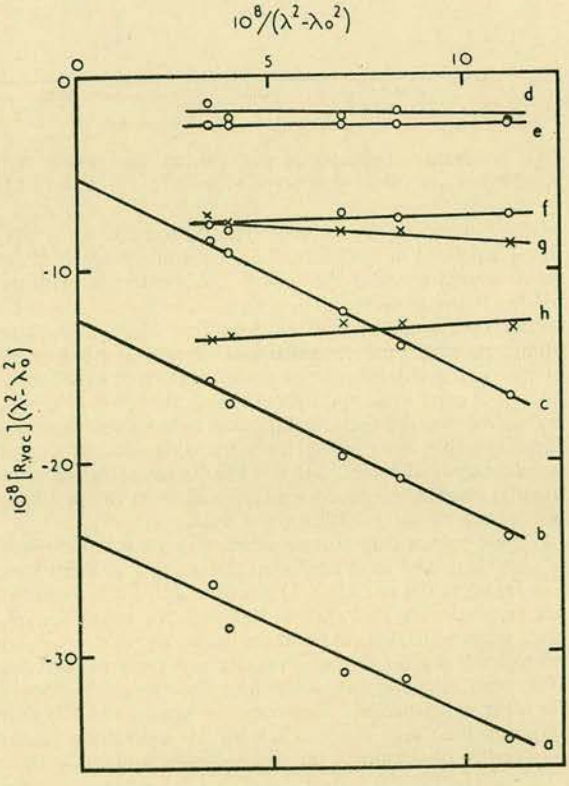

FIG. 2. — Dispersion of optical rotation for various solid films.

a, poly-L-alanine; *b*, polyalanine L/(D + L) = 0·90; *c*, polyalanine L/(D + L) = 0·80; *d*, polyalanine L/(D + L) = 0·67; *e*, *Bombyx mori* silk (from aqueous solution); *f*, *Bombyx mori* silk (from dichloracetic acid); *g*, potassium salt of poly-L-glutamic acid; *h*, lysozyme.

is very inexact, it is interesting to note that the values are all positive, that they increase at first with diminishing wavelength and then become stationary as though passing through a maximum before diminishing again—exactly as has been observed with solutions of α-polypeptides. The value of b_0 for the *meso* polymer (subject of course to considerable uncertainty) is $-560°$. The points corresponding to L/(D + L) equal to 0·67 do not lie on the linear part of the curves, and on plotting $[R_{vac}](\lambda^2 - \lambda_0^2)$ against $1/(\lambda^2 - \lambda_0^2)$ a graph of nearly zero slope is obtained (fig. 2*d*). This shows that b_0 is nearly zero, and the anomalous dispersion of the helix is absent.

The infra-red spectra of the polyalanine specimens shown in fig. 3 are in complete agreement with the deductions made from the optical rotation measurements. In the spectra of polymers of 1·0, 0·9 and 0·8 L/(D + L) composition, the bands at 893 and 906 cm^{-1}, known to appear in α-poly-L-alanine [8] are well-marked. The 906 cm^{-1} band is particularly important, since it appears to be associated with a crystalline arrangement of α-helices. In the spectrum of polyalanine of 0·67 L/(D + L) composition, this band is absent. There is, however, a strong band at about 966 cm^{-1}, which shows the presence

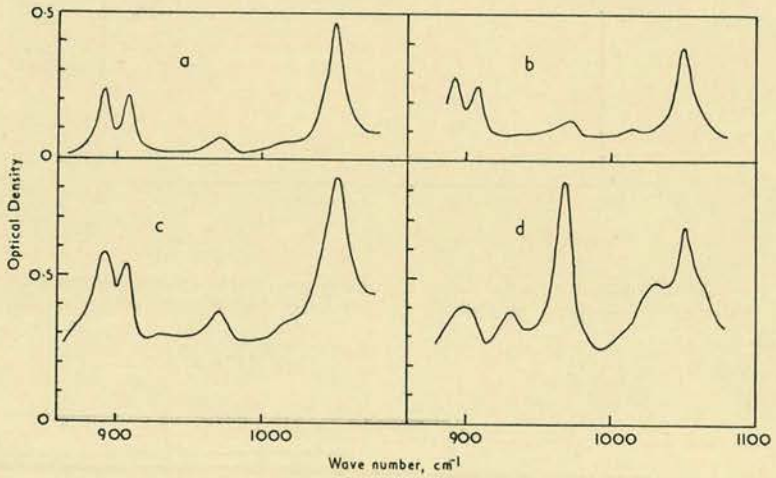

FIG. 3.—Infra-red spectra of polyalanine films whose optical rotation is given in fig. 1. *a*, L/(D + L) = 1·0; *b*, L/(D + L) = 0·9; *c*, L/(D + L) = 0·8; *d*, L/(D + L) = 0·67.

of the β form of polyalanine. The carbonyl band in the spectrum of a thin film shows the sharp band at about 1630 cm^{-1} characteristic of β polypeptides, as well as a broad band centred at about 1655 cm^{-1}. This coincides with the wave-number of the carbonyl mode of simple synthetic polypeptides in the α-helix form, but as will be shown in the section on the structure of water-soluble silk, it also occurs in the spectrum of an amorphous, possibly random coiled arrangement of polypeptide chains. Since the dispersion of the optical rotation shows conclusively that α-helices are absent in the film with 0·67 L/(D + L) composition, it appears likely that part of this polymer film is randomly coiled. It must be realized that the difference in behaviour between this polyalanine and the other three may not arise solely from the difference in the L/(D + L) ratio. The molecular weight may be different, and in addition it was found necessary to use a quick precipitant (methyl alcohol) to produce suitable films in the one case, whereas with the other three polymers a slower precipitant was used.

These results may be compared with some which were obtained earlier for solutions of polyalanine.[6] In chloroform containing 1 % dichloracetic acid the rotations are positive for all values of L/(D + L) in excess of 0·5 and the form of the curve shows that helices are present. The fact that we find *negative* values for $[R_{vac}]$ in the solid film shows the great effect which the environment has on the value of a_0, an effect which has been observed (in a much smaller degree) by Yang and Doty in solutions of poly-γ-benzyl-L-glutamate. It is therefore particularly interesting that the values of b_0 should be similar to those found for other polypeptides. This confirms Moffitt and Yang's contention that b_0 is much more invariant than a_0. The new results also extend the validity of a conclusion drawn from our earlier observations on poly-γ-benzyl glutamate that solvent effects on the optical

rotation of an α-helix of *meso* composition are small. Thus this quantity, given by the intercept on the *y*-axis of fig. 1 has the value 70° for light of wavelength 5780 Å, for the solid polymer. The corresponding value (for light of not greatly different wavelength 5893 Å) for *meso* polyalanine in chloroform containing 1 % dichloracetic acid [6] is about 80°.

From comparison of the X-ray diffraction pattern of solid films of α-poly-L-alanine with the optical transform of the α-helix, it has been shown that the right-handed helix is the dominant one (Elliott and Malcolm [9, 10]). It was at first believed that the negative value of b_0 found in measurements of optical dispersion in some synthetic polypeptides was evidence of this sense in solutions, but it appears doubtful whether this is a valid conclusion.[5] However, the fact that similar values for b_0 are found in polyalanine films and in a number of solutions of L-synthetic polypeptides must surely mean that the right-handed form is the stable one for the L-enantiomorph. The linear part of the plot in fig. 1 shows that for values of L/(D + L) of 0·8 and over, the left-handed form in solid polyalanine is not present to a significant extent.

ALKALI SALTS OF POLY-L-GLUTAMIC ACID

Films of the sodium and the potassium salts of poly-L-glutamic acid were made by dissolving the poly-acid in aqueous solution containing the stoichiometric amount of alkali, to give solutions of about 13 % concentration (w/v). The solutions, after filtering, were cast on glass plates at about 40°C in a dry air stream. The resulting films are very hygroscopic and it was found desirable to store them in a warm desiccator until a sufficient number had been made. They were then quickly transferred, still warm, to the polarimeter tube which contained phosphorus pentoxide. After measurement, the further manipulation for determining film thickness was done over a warm plate, and the films were well dried at 70°C before weighing in a closed bottle. Fig. 2(*g*) shows the results obtained for the potassium salt; for the sodium salt almost identical results were obtained.

The small value of the slope of the line is indicative of the absence of any considerable fraction of the polymer in the α-helix configuration. Films of both polymer salts of suitable thickness for infra-red measurement were prepared under similar conditions to those described above, and measured. The carbonyl stretching mode was found at 1658 cm^{-1} in both cases, hence these results furnish a second example of a polypeptide (in this case of simple composition) in which a carbonyl band near 1660 cm^{-1} is not associated with the helix form.

WATER-SOLUBLE SILK

Aqueous dispersions of *Bombyx mori* silk (made for instance by dissolving the silk in aqueous lithium bromide and dialysing out the salt) may be used to cast films which are soluble in water. Some years ago it was found that the spectra of these films have a carbonyl absorption band at 1660 cm^{-1} and since this same band is found in the spectra of synthetic α polypeptides it was suggested that water-soluble silk was in the α form (Ambrose, Bamford, Elliott and Hanby [11]). At this time, although the α-helix had been proposed by Pauling and Corey [12] its validity had not been established. The α-helix is now known to be a stable form of the synthetic polypeptides, but although good evidence of this form has been found in water-soluble *Antherea mylitta* and *Anaphe moloneyi* silks (from X-ray diffraction rings in both materials, and from infra-red spectra in the former) it has not been found in films of water-soluble *Bombyx* silk (Elliott and Malcolm [13, 14]). The evidence against the α-helix form for this last silk was, however, negative in character, and it appeared that a more positive indication could be obtained from measurements of optical rotation. Dilute aqueous solutions of *Bombyx* silk have been shown to be in a random coil form, and the α-helix form has been found in solutions in a suitable mixture of ethylene dichloride and dichloracetic acid.[4]

Films were cast by placing a few drops of the aqueous dispersion on glass plates heated to about 70°C on a small rotating table, and drying in a stream of dried, filtered air. Such films are not usually completely soluble in water again after drying, and for good solubility the films should be dried on mercury at 100°C. This is not practicable for films required for optical rotation measurement. The infra-red spectra of silk films dried on a solid substrate at 70°C are almost identical with those of water-soluble films except that a very small shoulder at about 1630 cm^{-1} shows the presence of a small amount of β material, which prevents the film from dispersing completely in water. The silk films were much too irregular in surface to use without an immersion medium, and for this purpose styrene was employed. The optical rotation was negative at all wavelengths, and dispersion was found to be of the normal type, which shows either that α-helices are not present or that there are equal numbers of right- and left-handed helices. In

view of what is known of the sense of the α-helix in simple polypeptides, this latter possibility can be excluded. The most sensitive test for the absence of " form " rotation is furnished by a plot of $[R_{vac}](\lambda^2 - \lambda_0^2)$ against $1/(\lambda^2 - \lambda_0^2)$. This gives a line of zero slope as shown in fig. 2 when λ_0 is given the value 2120 Å, hence the coefficient b_0 is also zero.

This result is an important one, and shows convincingly that a carbonyl absorption band at 1660 cm^{-1} does not necessarily indicate an α-helix form, even in a material which can under some circumstances take this configuration. The zero value for b_0 shows absence of " form " optical rotation. Polypeptides in the extended β form may have zero b_0, as is shown by fig. 2(*d*) and (*f*). However, the amount of β material present in silk films prepared from aqueous solution is insignificant, and a random arrangement seems likely. In air-dried silk films, an appreciable amount of water is present and it is likely that many peptide groups are hydrogen-bonded to water molecules. However, on heating such silk films to 70°C in a closed cell containing P_2O_5 for a number of hours much of this water is removed. The infra-red spectrum shows no trace of a free NH band and the carbonyl absorption band remains at 1660 cm^{-1}; hence it must be concluded that the hydrogen-bonding capacity of the silk is satisfied by intra- or inter-chain bonds. This presumably random-coiled form in the solid state must be considered as a stable form of silk, and one which may well be found in other dry proteins.

Since it is now established that neither aqueous, dilute dispersions of *Bombyx mori* silk nor the films cast under suitable conditions from such solutions contain α-helices, it is most unlikely that aqueous dispersions would have the helical form at some intermediate concentration. This conclusion casts considerable doubt on the claim to have established the existence of the α-helix as a major component in such dispersions by methods based on small-angle scattering (Kratky, Sekora and Pilz [15]).

LYSOZYME

It was found possible to cast films of lysozyme from aqueous solution and to measure the dispersion of the optical rotation, and also the infra-red spectrum in the region of the carbonyl stretching mode on specimens prepared under as far as possible identical conditions. A rather broad band centred at 1660 cm^{-1} was observed, with no indication of any peak or shoulder at 1630 cm^{-1}. The plot of the optical rotation measurements is shown in fig. 2(*h*), and gives no indication whatever of the kind of dispersion characteristic of α-helices. It is reasonable to conclude that the polypeptide chains in lysozyme films are neither in the extended (β) nor in the α-helix form. Lysozyme is capable of forming single crystals, but it by no means follows that in our films the polypeptide chains are in the same form as in crystals.

CONCLUSION

Since a polypeptide chain is capable of forming internal and external hydrogen bonds in a number of different folds, it is perhaps not surprising that polypeptide and protein films can be made in which neither the α-helix nor the extended β form are detectable, and this is what our experiments show. It is not known whether in such cases there is any degree of order in the films, and it may be that they are simply disordered states. Whatever the state of the polypeptide chains in these films, it is clear that they cannot be distinguished from α-helices by observations of the frequency of the C=O band alone.

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A film balance for use with the Langmuir trough

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Abstract. The force exerted by a monolayer on a flexible metal strip is measured by observing the deflection of the strip with a microscope. The apparatus is simple and fairly robust. A sensitivity of 0·03 dyn cm^{-1} is readily obtainable.

1. Introduction

Various types of film balance have been described for studying molecular monolayers spread on a Langmuir trough (see, for example, Davies and Rideal 1961, pp. 220–2). These all employ a torsion head to measure the force on a barrier in the surface. The barrier consists wholly or in part of fine threads floating on the surface and these may cause leaks. The instrument to be described replaces this arrangement with a comparatively rigid barrier made from a thin bent metal strip mounted with its lower edge in the interface. After the water surface has been swept clean the device is simply placed in position and the deflection of the barrier, caused by compressing a monolayer on one side of it, is measured with a microscope focused on its upper edge.

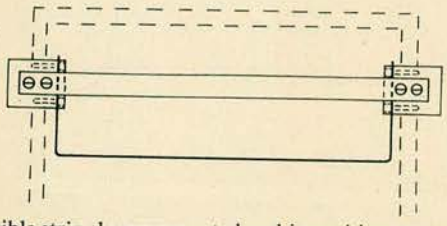
The apparatus was originally developed to study reactions in monolayers under a controlled pressure and then to remove them for spectroscopic or other examination. It has been found that condensed monolayers of various types can be removed by compressing them between two parallel barriers so that they collapse. A plate is then drawn along the gap between the barriers and the monolayer can usually be removed as a narrow strip of material across the plate. The comparative rigidity of the flexible strip (the second barrier being the usual strip of plate glass) makes this procedure more simple than use of a floating barrier.

Used simply as a film balance, the apparatus has been found to be very straightforward and it should be particularly useful for undergraduates, since in the normal laboratory class there is seldom time to operate successfully the usual type of instrument. There would appear to be no funda-

mental problem in adapting it for use at a liquid–liquid interface or increasing its sensitivity.

2. Description

Two simple geometrical forms of strip have been considered—a straight strip with a narrow 'V' in the middle, and the arrangement shown in the diagram where the strip forms three sides of a rectangle. Both arrangements have a similar sensitivity, but the former is liable to buckle if the strip is made of a heavy metal such as platinum on account



Flexible strip shown mounted and in position on trough (broken outline)

of the weight of the 'V' in the middle. For most purposes 0·003 in. thick phosphor bronze strip ¼ in. wide is satisfactory. This should be protected by a thin coating of hard paraffin wax. For work where traces of metal ions might affect the system, the strip can be made of hard-rolled platinum–2% rhodium; in this case waxing is unnecessary. If a plastic strip is used trouble may be experienced in gradual drift of the zero or movements caused by swelling. As an alternative a metal strip can be coated with a film of polyvinyl fluoride 0·0005 in. thick (obtainable from du Pont Company) by folding it over the lower edge of the strip and cementing it to the metal before bending the strip.

The strip is mounted by clamping it to blocks of unplasticized plastic using counter-bored screws, preferably of nylon. The screw holes drilled in the strip should have plenty of clearance so that the lower edge of the strip can be set flush and coplanar with the lower surfaces of the blocks. The clamping pieces should not project below the lower edge of the strip. A strong metal bar bolted across the top surfaces of the blocks holds them apart so that their inner edges overlap the inner edges of the trough by about 0·5 cm. Care should be taken that the top of the trough is flat and the blocks resting on it are coplanar, otherwise the device will rock when placed in position and leaks may occur. After clamping the strip the blocks should be given a coating of paraffin wax. Provided that the water level is just above the edges of the trough, a good seal should be obtained as soon as the barrier is placed in position.

The deflection of the centre of the strip can be measured with either a fixed microscope with a micrometer eyepiece or, preferably, a travelling microscope with a cross-wire in the eyepiece and a micrometer drive. In this latter case a simple holder for a microscope tube can be made, incorporating a parallel spring-strip horizontal movement driven by a micrometer anvil; about 5 mm horizontal movement is sufficient. The microscope should be very firmly mounted and we have found the large adjustable stand made by C. F. Palmer (London) Ltd. satisfactory. The vertical screw movement enables the microscope to be completely removed for the preliminary stage of sweeping clean the water surface; it is also sufficiently sensitive for focusing a 10× objective on the edge of the strip. Adequate illumination is obtained by using a low power lamp shining obliquely on the strip if the image of the filament is roughly focused on the strip edge.

With a strip of phosphor bronze, 0·003 in. thick and ¼ in. wide formed into a barrier with a length of 14·3 cm and side arms 2·8 cm long, a displacement of 1 mm at the centre of the strip corresponds to a force of about 16 dyn cm^{-1}. This can be conveniently measured with the micrometer movement to $\pm 2\ \mu$m, using a 10× objective and a 10× eyepiece, giving a sensitivity of $\pm 0{\cdot}03$ dyn cm^{-1}. For a similar strip made of hard-rolled platinum–2%rhodium, 1 mm deflection corresponds to about 23 dyn cm^{-1}.

3. Calibration

There are two methods of calibrating the balance: either indirectly using a monolayer of known properties, or by measuring the deflection of the barrier for a point load applied at the centre and converting this to an equivalent uniformly distributed load. Though flexure devices often obey simple theory only approximately, this latter method appears satisfactory unless high absolute accuracy is required, when it might be better to use a torsion balance and float.

It can be shown that for a point load of W dyn applied at right angles at the centre of the strip, the displacement d is given by

$$d = \frac{Wl^3}{Ebt^3}\left(\frac{4 + 4a}{8 + 2a}\right)$$

where E is Young's modulus, t the thickness of the strip and b the breadth. The length of the barrier between the two side arms is taken as $2l$, and a is the ratio of the length of the side arm to l.

For a distributed load w per unit length applied to the length $2l$ the displacement is

$$d = \frac{wl^4}{Ebt^3}\left(\frac{4 + 5a}{8 + 2a}\right).$$

Thus the distributed load which gives the same displacement as a point load W is given by

$$w = \frac{W}{l}\left(\frac{4 + 4a}{4 + 5a}\right).$$

In the derivation of these formulae it has been assumed that the loads exerted on the side arms by the monolayer can be neglected (which can be shown to be reasonable) and that the displacements are small. For the phosphor bronze strip with the dimensions above the displacement was found to increase linearly with the applied load up to $d = 1{\cdot}5$ mm. The calibration with a point load has been checked directly with a monolayer by measuring the pressure of the monolayer with a float and torsion balance. The two methods agreed to within 2%; for a strip of platinum–2% rhodium with the same dimensions there was a 6% difference. A value of a of about 0·4 appears satisfactory; if a smaller value is used the formulae are less accurate, since the assumption that the displacement is small is no longer true and the device is stiffer than theory predicts.

A horizontal load for calibration can be applied by linking the strip with a fine straight wire to the lower end of the vertical arm of a T-piece made of stiff wire. This is pivoted to rotate in its plane about the junction of the T by being mounted on a fine torsion wire. Loads can then be applied with a torsion head or by weights on the horizontal arms.

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Conformation of Synthetic Polypeptide and Protein Monolayers at Interfaces

SYNTHETIC polypeptide monolayers at the air–water interface are generally thought to be in extended-chain conformations[1] though Bamford *et al.*[2] have pointed out that the experimental data can often be interpreted as indicating the presence of the α-helix[3]. Similarly, protein monolayers are usually considered to be denatured with the polypeptide chain fully extended[4].

To investigate the possible presence of the α-helix at the air–water interface, deuterium-exchange in N-deuterated synthetic polypeptide monolayers has been studied. In solution peptide deuterium exchanges for hydrogen rapidly when the peptide groups hydrogen-bond to solvent molecules, but exchange is very slow when the polypeptide is in the α-helical conformation[5]. Slow exchange in monolayers might similarly be an indication of the presence and stability of the α-helix. The polymers used were poly-D-α-amino-*n*-butyric acid, poly-DL-leucine, poly-γ-methyl-L-glutamate, poly-γ-ethyl-L-glutamate and poly-γ-benzyl-L-glutamate, all prepared by Mr. W. E. Hanby, of Courtaulds, Ltd., Maidenhead, to whom I am indebted. All the polymers were spread from dilute solution in chloroform containing 8 per cent v/v O-deuterated dichloroacetic acid, with the exception of poly-DL-leucine, where benzene was used in place of chloroform.

After being spread as monolayers on N/100 hydrochloric acid at 20° C for 10 min the films were swept off the surface, dried and examined by infrared spectroscopy. The spectra in all cases showed very little deuterium-exchange and were typical of polypeptides in the α-helical form. Exchange was observed in similar experiments using a substrate of N/100 sodium hydroxide though it was not always complete, and again the spectra were consistent with the presence of the α-helix. A series of experiments with poly-γ-methyl-L-glutamate has shown that the exchange depends on the time the film is on the substrate and it is favoured by a high *p*H and temperature. The spectra show that these same conditions also eventually lead to the formation of the extended β-conformation. These results are all con-

sistent with the general presence of the α-helix which becomes less stable on alkaline substrates so that ultimately the β-conformation may be produced.

Measurements of the area per residue on a Langmuir trough supported this conclusion and agreed within 10 per cent of the areas calculated from the closest packing distances obtained by X-ray diffraction observations of the polymers in the α-helical form. The surface potentials observed varied from 340 mV to 590 mV on the acid substrate and were somewhat less on the alkaline. The α-helix can have no appreciable dipole-moment at right-angles to its axis unless the side-chains are polar and are arranged asymmetrically on the water surface. If the conclusions presented here are correct this must mean that, at least for the polymers with hydrocarbon side-chains, the surface potential arises mainly from adsorption of water molecules on to the helix.

Since the α-helix is probably the stable conformation of these polymers in a non-hydrogen-bonding environment, a further conclusion is that this will also be their probable conformation at the oil–water interface. Doty[6] has shown that the helical content of proteins in solution is increased by decreasing the hydrogen-bonding capacity of the environment. In the light of the results given here, this suggests that at least the regions of proteins where the side-chains are mainly hydrocarbon will be in a helical conformation at the lipid–water interface. Quite possibly the unfolding observed with proteins at interfaces is often a loss of the tertiary structure.

The possibility that the α-helix is stable at the lipid–water interface is of considerable importance in relation to the biochemical activity of interfaces. In contrast to extended chain conformations, the all-round arrangement of side-chains in relatively well-defined positions could lead to specific interactions with adjacent protein, with the components of the lipid and with the aqueous phase.

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Structural Changes and Molecular Forces in a Compressed Synthetic Polypeptide Monolayer

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It has been found that structural changes take place when a monolayer of poly-γ-methyl-L-glutamate is compressed. The first stage is the regular collapse of the α-helices in the monolayer to form a bilayer. Further compression appears to cause the formation of a third layer of molecules before the film starts to fold and collapse completely. During this process the molecules become aligned with the mean direction of their axes at right angles to the direction of compression. From the force required to produce a bilayer and the angle of contact of the polymer/liquid interface, the work of cohesion of the polymer has been calculated. Taking into account the molecular structure of the bilayer, and making certain assumptions, the mean work of cohesion per mole of residue pairs has been found to be 3·6 kcal.

EVIDENCE has been obtained to show that the monolayers of a number of synthetic polypeptides are in the α-helical conformation at the air/water interface (Malcolm[1,2]), whereas previous work based on the traditional methods of surface chemistry has led to extended conformations being proposed (Cheesman and Davies[3]). The hypothesis, that for certain synthetic polypeptides the α-helix is stable at the air/water interface, has been based not only on their surface chemistry but also on measurements of deuterium exchange rates and examination of collapsed films by infra-red spectroscopy. Further evidence (to be published), which supports the original conclusions, has since been obtained from electron diffraction examination of collapsed monolayers.

The α-helix is rigid and rodlike by comparison with most polymer conformations, so that the molecules in a monolayer must pack together in parallel groups. It appears that, as a consequence of the local order existing in the groups, when the molecules are compressed a bilayer is formed as a regular process. Since the pressure required to form the bilayer is dependent on the cohesive forces of the molecules of the polymer and the substrate, these observations lead to a new experimental approach to understanding molecular cohesion.

Results will be given here for poly-γ-methyl-L-glutamate to illustrate the process. A full account of the structure and properties of synthetic polypeptide monolayers, particularly in relation to their biological interest, will be given elsewhere.

EXPERIMENTAL

Poly-γ-methyl-L-glutamate with a reported intrinsic viscosity of 1·04 in dichloracetic acid was used, prepared by Yeda Research and Development Co. Ltd. The molecular weight was estimated by the makers to be

260 000 from viscosity measurements by comparison with measurements on poly-γ-benzyl-L-glutamate. Standard solutions for spreading monolayers were made by dissolving about 10 mg in 1 ml dichloracetic acid which was then made up to 10 ml by addition of chloroform. About 0·03 ml of solution was spread on a clean water surface in a fused quartz Langmuir trough. The work was greatly facilitated by the use of a new type of film balance (Malcolm and Davies[4]) consisting essentially of a bent metal strip with its lower edge in the interface. The deflection of the strip, which is proportional to the force on the film, is measured with a microscope. With a platinum–2% rhodium strip 0·003 in. thick, ¼ in. wide, 1 mm deflection corresponds to a force of 23 dyne cm^{-1} on the monolayer. The monolayer was compressed by a barrier moving continuously at the rate of 2 mm/min. All other experimental details, which followed the usual procedures of surface chemistry, were as given previously[2].

RESULTS AND DISCUSSION

Surface area measurement

The force/area curve for a monolayer spread on 0·01 N hydrochloric acid is shown in *Figure 1*. Similar results were obtained using a substrate

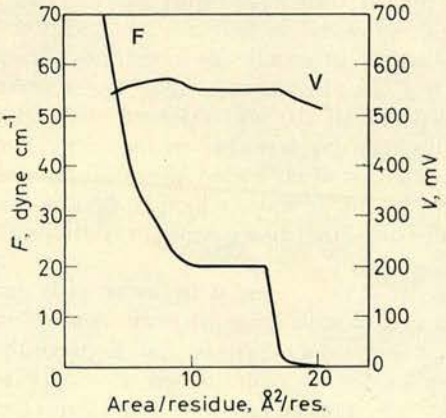

Figure 1—Force/area (*F*) and surface potential measurements (*V*) for a monolayer of poly-γ-methyl-L-glutamate on 0·01 N hydrochloric acid, 20°C

of distilled water. The monolayer, as prepared, is stable over many hours and the force/area curve unaffected by doubling the rate of compression. Extrapolation of the first steep rise of the force/area curve to zero pressure gives an area per residue of 17·5 ± 0·3 Å². At a pressure of 20 dyne cm^{-1} there is a yield point and under slow continuous pressure the film sustains an almost constant force until, at about 10 Å²/residue, the force on the film again rises until at 70 dyne cm^{-1} the film finally collapses. This is shown by irregular movements and rapid drifting of the film balance.

The area per residue of 17·5 Å² may be compared with 17·9 Å² calculated assuming that the molecules are in the α-helical conformation and packed at the same distance as in the solid state in a hexagonal cell with $a = 11{\cdot}95$ Å

and an increment of 1·5 Å per residue along the molecular axis[5]. Precise agreement is not to be expected since the packing of the molecules in a monolayer is not necessarily the same or as perfect as in the crystalline state. This evidence and measurements of deuterium exchange rates (to be published) are consistent with the assumption that the molecules are in the α-helical conformation. Without this hypothesis it is difficult to explain many of the observations.

An area of 10 Å^2/residue is too small to accommodate any reasonable conformation of this polymer in a monolayer and the plateau must therefore be associated with molecules being forced out of the surface and forming a second layer. The plateau does not quite extend to half the condensed area of a monolayer, which suggests that a proportion of the molecules in the monolayer are not in a regular arrangement and therefore require additional work in order to force them out of the surface. At this stage some might start to form a third layer. Over the extent of formation of the plateau, however, the collapse is probably propagated from points where isolated molecules are forced out of the surface. Adjacent molecules in the lower layer will then be in an asymmetric field and pulled upwards. They in turn will act on further molecules. The plateau has not, in any case so far examined, been found to extend to less than half the area of a close-packed monolayer. Its length and the pressure required to form it under given conditions appear to be quite reproducible and a characteristic of the polymer.

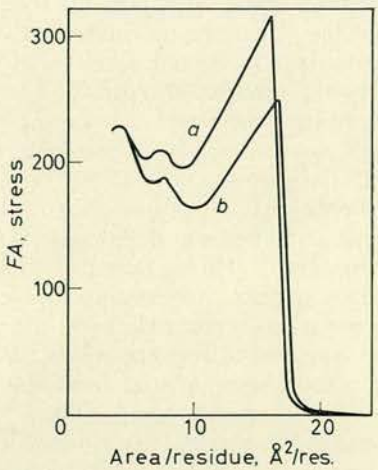

Figure 2—(*a*) Stress/area diagram derived from *Figure 1*, and (*b*) the effect of the addition of ½ per cent v/v isopropanol

The conventional force/area curve for a monolayer, as in *Figure 1*, is probably not the best way to present experimental data in which changes in the mechanical strength of the structure may be taking place. An alternative way is shown in *Figure 2*, in which the product of the force F (in dyne cm^{-1}) and the area A (Å^2/residue) is plotted against A. The product FA represents a quantity proportional to the force per unit cross-

sectional area (i.e. stress) that would be applied to a homogeneous material which increased uniformly in thickness as the area of the film decreased, so that the density remained constant. The deformation of the film as measured by the decrease in area, may be considered to represent the strain on the film and thus *Figure 2* is a form of stress/strain diagram. The collapse of the film to a bilayer is now shown as a continuous decrease in the stress sustained by the film until the process is complete. A further collapse is also indicated giving rise to a minimum at about 6 Å^2/residue, which is probably associated with the formation of a third layer of molecules. The peak of the curve at about 4 Å^2/residue is caused by the onset of folding and collapse of the film, though it may be mentioned that with poly-L-norleucine a further peak is observable before collapse, suggesting the formation of a fourth layer of molecules.

The stress, as calculated from the product FA, is only strictly meaningful when the film is of uniform thickness, i.e. after the formation of an integral number of layers. Nevertheless the fact that after what has been interpreted as a regular collapse is followed by an increase in the stress, as calculated, is a good indication that a transformation giving rise to an ordered structure has taken place. It will be seen that this type of analysis enables information to be obtained which may otherwise be hidden in the steep portions of a force/area graph. Finally, it should be pointed out that since the molecules are becoming orientated during compression (see below) a more detailed analysis of the data should take this into consideration.

Surface potential measurements

Above about 20 Å^2/residue the surface potential is non-uniform showing that the monolayer is condensed. Below 20 Å^2/residue it rises in proportion to the fractional decrease in area down to 17 Å^2/residue (*Figure 1*). This suggests that the dipoles contributing to the surface potential are not becoming reorientated, but simply pressed closer together. Between the yield point and 10 Å^2/residue the potential remains almost perfectly constant.

In the case of poly-L-alanine the surface potential arises entirely from a net reorientation of the water molecules consequent upon spreading the monolayer[2]. This is because, on account of its symmetry, the backbone of a long α-helix can have no dipole moment at right angles to its axis. If, however, as is the case here, the polymer has flexible side-chains, besides the component from the water, an additional contribution is to be expected in a monolayer arising from the dipoles in the side-chains. These will produce a direct component if the dipoles in the side-chains are not arranged in a symmetrical manner so that they cancel out, and an indirect component arising from interaction of some of the side-chain dipoles with the underlying water. It is not possible to judge how big these contributions are, but they appear largely to cancel each other out since the resultant potential is comparable in magnitude to poly-L-alanine. The second layer of polymer might reasonably be expected to have its side-chains in an approximately helical arrangement so that neither the side-chains nor the peptide groups of the second layer can give rise to a potential. If the horizontal plateau in the force/area curve is attributed

to the steady formation of a bilayer of α helices, the remarkable constancy of the surface potential below 17 Å^2/residue can therefore be understood. On the other hand, if the structure of the first layer were an extended polar structure, which underwent a reorientation or conformational change at the yield point, the surface potential would in general be expected to increase or decrease on further compression.

The mechanism of bilayer formation

The pressure required to form a bilayer is altered by not more than one per cent (approximately the limit of relative accuracy for two consecutive experiments) by raising the temperature from 20°C to 30°C or by changing the substrate from 0·01 N hydrochloric acid to distilled water. However, addition of half of one per cent by volume of isopropanol to the trough reduces the pressure required by 5 dyne cm^{-1} (*Figure 2*). This suggests that the pressure necessary is dependent on the work of adhesion of the monolayer to the substrate and gives a clue to the underlying physical process. The work done (if the conversion were 100 per cent) in forming 1 cm^2 bilayer at the observed pressure of 20 dyne cm^{-1} is clearly 20 ergs. Since this involves the removal of 1 cm^2 of monolayer from the water surface, energy must be supplied equal to the work of adhesion W_{SL} of the monolayer to the substrate. This may be calculated from Young's equation

$$W_{SL} = \gamma_{LA}(1 + \cos\theta)$$

where γ_{LA} is the surface tension of the liquid with respect to air and θ the angle of contact for the polymer/liquid interface[6]. θ has been measured by casting a thin clear film of polymer on a glass plate and using the usual dipping plate technique[7]. The value obtained, once the polymer has been dipped below the surface, is 58° ±2° for both distilled water and 0·01 N hydrochloric acid. Addition of half of one per cent isopropanol lowers θ by about 2°. A value 5° higher is obtained if the surface has not previously been wet; this type of hysteresis is not uncommon, but the reason for it in this instance is not known. Taking the value of 58° and $\gamma_{LA} = 72{\cdot}8$ dyne cm^{-1} the work of adhesion of the polymer to water is approximately 111 erg cm^{-2}. Since the mechanical work done in compressing the film is only 20 erg cm^{-2}, it follows that the additional energy for the formation of the bilayer, 91 erg cm^{-2}, is derived from the work of cohesion of the second layer of molecules on top of the first.

It is convenient to introduce here the term 'residue-pair' when discussing a bilayer meaning one residue from each layer. If the bilayer is taken to have a projected area per residue pair of 17·9 Å^2, from the figure above the work of cohesion per mole of residue pairs is 2·35 kcal. If it is assumed that the molecules pack in the same manner as in an infinite hexagonal cell, with the *a* and *c* axes parallel to the surface, two thirds of the total number of residues in an infinite structure may be considered to be involved in holding adjacent planes together (the remaining one third hold the molecules together within the plane as, we assume, in a monolayer). Thus the mean work of cohesion of all the residues in an infinite hexagonal

structure is 3·5 kcal/mole of residue pairs (subject to a small correction, see below).

This analysis neglects changes in the entropy of the molecules as a result of the formation of the bilayer. Since both before and after the transition they are presumed to be packed in parallel groups, the entropy change involved is likely to be very small and is neglected.

The assumptions concerning the way the molecules pack when the bilayer is formed require further justification since (*a*) when the bilayer starts to form, the monolayer is under pressure and occupying 16 Å^2/residue rather than 17·9 Å^2/residue as in an unstrained hexagonal cell, and (*b*) it has been assumed that in an unstrained bilayer the packing is hexagonal rather than, say, orthorhombic. A correction for (*a*) can be estimated from the additional work involved in compressing a bilayer from 17·9 Å^2/residue pair to 16·0 Å^2/residue pair. If this is taken as approximately twice the work done in compressing a monolayer over the same monolayer areas, the work done on a bilayer is 2 erg cm^{-2}. Thus the mechanical work done on a monolayer to convert it to an unstrained bilayer is reduced from 20 to 18 erg cm^{-2}. This increases the calculated work of cohesion to 3·6 kcal/mole of residue pairs. Assumption (*b*) is reasonable since there is fairly good agreement between the observed area per residue for an unstrained monolayer and the area expected assuming the same intermolecular distance as in a hexagonal cell, and also it is consistent with the results of electron diffraction examination of the collapsed multilayer (see below).

A further assumption is that the work of cohesion only involves nearest molecules, so that the work of cohesion between the planes of a bilayer is assumed to be the same as between similar planes in an infinite structure. A similar point arises in applying the angle of contact derived from measurements on a cast film of polymer to the calculation of the work of adhesion of a monolayer. In the absence of ionic interactions these are probably good approximations. A more difficult problem is to be sure that the angle of contact measured on the surface of a cast film is strictly applicable to a monolayer, where the structure of the film may be rather different. This may not be a serious limitation, but in the absence of any obvious experimental approach to justify this point, it should be borne in mind.

The corrected value for the work of cohesion per residue pair, 3·6 kcal/mole, appears reasonable. Interpenetration of the side-chains, for which there is direct electron diffraction evidence[8], must make an important contribution to this figure as a result of dipole and van der Waals interactions. However, it is not possible to suggest precise conformations remembering that the helices do not have an integral number of residues per turn and are probably packed with the peptide sequence of individual molecules running randomly in either direction with respect to the molecular axis. The figure is therefore an average value for the diversity of packing arrangements which must be present.

Observations using electron diffraction

If a film is further compressed after the point of final collapse, wrinkles develop which can be seen by oblique illumination of the surface. It can

then be removed as described previously[2] or picked up on electron microscope grids. The grids are dropped on to the film and the film and grids are then lifted off by means of a piece of Perspex, to which the film adheres, being gently placed on the surface. Optical microscope examination shows that in all cases so far examined, the molecules have become orientated with the mean direction of orientation at right angles to the direction of compression. Electron diffraction patterns (to be published) obtained with an Associated Electrical Industries E.M.6 electron microscope show clearly the main diffraction features of the α-helix including the meridional reflection at 1·5 Å. In the particular case of poly-γ-methyl-L-glutamate it is often possible to observe what approximates to a 'single crystal' diagram in that the diffracting crystals in the field (diameter 6μ) are not only all orientated with their molecular axes (c axis) in approximately the same direction but also with an a axis in the plane of the specimen. The diffraction pattern has therefore fewer reflections than the usual type of fibre diagram. This is good support for the hypothesis that the molecules are in the α-helical conformation in the monolayer, and for the interpretation of the surface chemistry data. It also suggests that the procedure used to prepare the specimens might be developed into a logical and precise method for preparing orientated polymer specimens for electron diffraction and other purposes.

CONCLUSIONS

Isemura and Hamaguchi have made observations on the surface chemistry of a number of polymers including poly-γ-methyl-L-glutamate. They appear to have observed a similar collapse phenomenon to the one described here in poly-DL-α-aminocaprylic acid and poly-DL-α-amino-capric acid[9], which, however, they attribute to a reorientation of the molecules and, in poly-γ-benzyl-L-glutamate and -DL-glutamate[10], without offering an explanation. Their data for poly-γ-methyl-L-glutamate appear to be seriously in error, possibly in part due to the use of pyridine as a spreading solvent. They consider the conformation to be that proposed by Ambrose and Hanby[11] for the α-structure, but in view of later work (Bamford, Elliott and Hanby[5]) and the work reported here, this seems unlikely.

It remains to be seen how general is the formation of a plateau in force/area curves of other polymers. So far, it has been found in the methyl, ethyl and benzyl esters of poly-L-glutamic acid, but only an inflection is observed in polypeptides with normal hydrocarbon side-chains unless the side-chain has four or more carbon atoms. It would, therefore, appear that the formation of a bilayer is facilitated by a measure of flexibility in the side-chains.

When it can be shown that the collapse of the polymer film is a regular process, as it is here, observations on synthetic polypeptide monolayers can evidently provide a direct quantitative experimental approach to understanding the cohesional forces between molecules. The information which might be obtained in this way is particularly important in relation to a proper understanding of the stability and conformational changes of proteins.

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Molecular structure and deuterium exchange in monolayers of synthetic polypeptides

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[Plate 4]

An investigation has been made of the structure and properties of synthetic polypeptide monolayers spread at the air/water interface. Two series of high molecular weight polymers have been examined, esters of polyglutamic acid and polymers with hydrocarbon side-chains. The structure has been investigated by measurement of the force/area relations and surface potentials with a Langmuir trough, and by measurement of the exchange rates of peptide deuterium. These direct methods have been supplemented by observations by infrared spectroscopy and electron diffraction on collapsed films removed from the surface. It is found that in all cases the properties of the monolayer are consistent with a structure consisting of condensed ordered arrays of α-helices. The collapse of a monolayer to form a bilayer causes either a plateau or an inflexion in the force/area curve. From the pressure required to form the bilayer and the work of adhesion between the polymer and water, obtained from Young's equation, the free energy of the polymer/vapour interface has been calculated. The deuterium exchange measurements show that exchange can take place in an intact α-helix, and how the exchange rate is influenced by the accessibility of the peptide group to water, the pH of the substrate and the hydrophobic nature of the side chain; the conclusions are important in the interpretation of deuterium exchange in proteins.

Introduction

Recent advances in the study of large molecules in the crystalline state have not been accompanied by corresponding improvements in methods for the study of systems with lower degrees of order. Nevertheless, in order to understand intermolecular cohesion, interfacial reaction mechanisms and the structure of biological membranes, it is essential to obtain a detailed picture of surface and lamellar structures. The standard methods of surface chemistry are of course valuable, but the information obtainable is restricted and in some cases interpretation of the data has been rather speculative. To obtain more information it is necessary to try to bridge the gap between classical surface chemistry and the usual methods of structural investigation.

In the work to be described, the standard methods of surface chemistry have been supplemented by using infra-red spectroscopy, deuterium exchange and electron diffraction, to give an understanding of the structure and behaviour of molecular monolayers of synthetic polypeptides spread at the air/water interface. These polymers have considerable intrinsic interest, and in addition they have been extensively investigated as model compounds for proteins. Their value in this respect has been limited in some instances, since many are insoluble in water. This limitation does not apply to the monolayer state and these materials provide an

opportunity to examine hydrogen bonding and hydrophobic interactions in the asymmetric environment of the water/polymer/air interface.

While a number of individual polymers have been previously investigated, it is of great value to be able to make comparisons to assess the effects of side-chain size and hydrophobic character from one polymer to another. Two series of polymers have therefore been used, three esters of poly-L-glutamic acid, and five polymers with hydrocarbon side-chains, poly-DL-leucine and four with unbranched side-chains containing one to four carbon atoms.

The primary aim of this work was to establish as precisely as possible the conformation of the polymers at the air/water interface. Cheesman & Davies (1954) concluded that monolayers of polypeptides consisting of non-polar residues are folded at the air/water interface with the side-chains alternately up into the air and down into the water, and Mishuck & Eirich (1955) discussed their results in terms of a similar extended chain conformation. At the oil/water interface the chains were all supposed to be free to enter the oil. Bamford, Elliott & Hanby (1956) found this model unconvincing and suggested that the α-helical fold of Pauling & Corey (1951) should be considered a possibility at the air/water interface. They pointed out that often the area per residue, measured with a Langmuir trough, agreed well with the area calculated from X-ray data on the polypeptide in the α-helical conformation. However, not all the published data agreed with this hypothesis; Cheesman & Davies found for poly-DL-leucine an area of 17 Å^2 per residue on 0·01 N hydrochloric acid but only 15 Å^2 on 0·1 N sodium hydroxide; Cumper & Alexander (1950) obtained 14 Å^2 per residue for poly-DL-phenylalanine and Isemura & Hamaguchi (1952) found 9·8 Å^2 per residue for poly-γ-methyl-L-glutamate, areas too low to accommodate α-helices. Higher areas around 17 to 18 Å^2 per residue can often be interpreted either as α-helices or extended chains. At the oil/water interface, the suggestion that the side-chains are all directed into the oil cannot be applied to polymers consisting of only one enantiomorph since, as has been pointed out (Malcolm 1965), with a planar *trans* amide group and the usual bond angles, conformations with the side-chains all directed to one side of an interface are impossible.

The surface potential has frequently been used to provide information about the orientation of polar groups at interfaces and Davies (1951, 1953*a*, *b*) related the surface potential of poly-DL-leucine to the orientation of the C=O dipoles. It has, however, always been recognized that, as a consequence of the way the potential is measured and defined, it includes an unknown contribution arising from the dipoles of the water (Adam 1941; Davies & Rideal 1961), and Alexander (1958) has pointed out in this context that attempts to relate the orientation of peptide dipoles directly to the surface potential can give rise to anomalies.

The foregoing shows the need to extend the standard methods of surface chemistry and to supplement them with further both direct and indirect methods. Structural studies by X-ray diffraction methods on monolayers are not of course possible, but by removing the monolayer a dry multilayer specimen can be prepared and examined by any of the usual methods. Astbury & Bell (1938) used this method to study monolayers of egg albumin and concluded that the dry film consisted of protein in the β-conformation. Others do not seem to have followed up this approach.

In the work to be described, X-ray methods have not been used since the quantity of material obtainable from a single monolayer is very small; it is, however, sufficient for examination by infrared spectroscopy. This method has added advantages, not only does the spectrum give information about the structure of both the crystalline and amorphous regions of the specimen, subjected only to vacuum drying after removal from the interface, but it has been found possible to use the absorption spectrum to measure the rate of deuterium exchange in the monolayer (Malcolm 1965). This provides a direct method for investigating hydrogen bonding in polypeptide monolayers and extends the methods pioneered by Linderstrøm-Lang and co-workers (Hvidt & Nielsen 1966) on proteins and related compounds in solution. Studies on model compounds in solution show that there is rapid exchange between peptide deuterium and hydrogen when the peptide group is free to hydrogen-bond to solvent water. In proteins there is usually a slowly exchanging component, and this is related to the conformation of the molecule. Detailed interpretation of the results from proteins is complicated and some of the problems will be discussed in the light of the results from monolayers, which are useful model systems in this respect. In applying this method to investigate the structure of a monolayer caution is necessary. However significantly different rates of exchange are to be expected in a monolayer composed of α-helices, compared with conformations in which the N—D groups are either hydrogen-bonded to the underlying water or exposed to the saturated water vapour above the interface.

When monolayers of the polymers considered here are collapsed and removed from the surface to provide specimens for infrared spectroscopy, they are found to be in the α-helical conformation (Malcolm 1962, 1965). This has now been confirmed by electron diffraction and together these are useful indirect methods. Nevertheless, there is always the possibility of a conformational change on removing a monolayer from the surface, and this is almost inevitable if it is stabilized, (e.g. by hydrogen-bonding) by direct interaction with the substrate. It is therefore essential to correlate conclusions drawn from indirect observations with those from direct methods. Thus, in the work to be described, while infrared spectroscopy and electron diffraction have shown beyond reasonable doubt that all the polymers after removal from the interface are in the α-helical conformation, pains have been taken to try to establish whether or not this is their stable conformation at the air/water interface.

Materials and methods

Materials

Poly-γ-methyl-L-glutamate and poly-γ-benzyl-L-glutamate were obtained from Yeda Research and Development Company Limited, Israel. The molecular weights were estimated by the makers from viscosity measurements to be 260 000 and 75 000 respectively. The following polymers were kindly provided by Mr W. E. Hanby of Courtalds Ltd, Coventry; where possible and when available in sufficient quantity for viscosity determinations, the reduced viscosity (1/base mole) measured at $\frac{1}{2}$ % concentration in dichloracetic acid is given in brackets: poly-D-alanine (21·6), poly-D-α-amino-*n* butyric acid (29), poly-L-norvaline, poly-L-norleucine, poly-DL-

leucine and poly-γ-ethyl-L-glutamate. These polymers had all been prepared to give high molecular weight material. The methyl-, ethyl- and benzyl- esters of poly-L-glutamic acid will be denoted by *PMG*, *PEG* and *PBG* respectively.

All solutions for spreading monolayers, except for poly-DL-leucine, were made by dissolving about 10 mg of polymer in 1 ml. dichloracetic acid and making up to a volume of 10 ml. by addition of chloroform; for poly-DL-leucine, benzene was used instead of chloroform. Where *N*-deuterated polymers were required, the dichloracetic acid was prepared in the *O*-deuterated form by repeated distillation from a mixture containing an excess of 99·8 % D_2O. Analytical reagent grade chemicals were used without further purification. Twice distilled water (the second time from alkaline potassium permanganate solution) was used in the Langmuir trough. Dichloracetic acid was purified by vacuum distillation.

Force/area and surface potential measurements

A fused quartz Langmuir trough 15 cm wide was used with a new type of film balance (Malcolm & Davies 1965), consisting essentially of a platinum/2 % rhodium strip $\frac{1}{4}$ in. wide, 0·003 in. thick bent to form three sides of a rectangle. Each end of the strip is clamped and mounted with the long dimension of the strip, 14·5 cm, at right angles to the sides of the trough. The lower edge of the strip is in the interface forming a flexible barrier across the surface. The deflexion of the strip, which is proportional to the force on the film, was found to take 23 dyn/cm for 1 mm deflexion of the centre of the strip. This was measured with a microscope fitted with a micrometer movement to $\pm$ 0·002 mm.

After the water in the trough had been repeatedly swept clean, the flexible barrier was placed in position and about 0·03 ml. of solution of polymer applied to the surface from an all-glass Agla micrometer syringe. The monolayer so formed was compressed against the flexible strip with a waxed glass barrier which moved forward continuously at the rate of 2 mm/min. Surface potentials were measured with a potentiometer coupled to a Lindemann electrometer and a 1 mC polonium air ionizing electrode above the surface; contact to the trough was made with a calomel electrode.

Infrared spectroscopy and measurement of deuterium

To remove polymer monolayers for examination by infrared spectroscopy, compression of the film was continued until the separation between the barriers was about 1·6 cm. The collapsed film could then usually be removed as a narrow strip of wet polymer, by drawing a barium fluoride plate across the trough between the two barriers. It was then gently blotted down and dried in a vacuum. For the measurement of deuterium exchange rates compression was manual, much more rapid, and as soon as the wet polymer strip was removed, it was rinsed with 0·01 N hydrochloric acid to reduce any further exchange to a minimum. In some instances the strip of polymer was found to be rather white as a result of the vacuum drying. This caused appreciable scatter of infrared radiation and could be avoided by applying to the dry film a drop of benzene, which rapidly evaporated, to leave a

compact clear film. This procedure was used only for deuterium exchange measurements.

Infrared spectra were obtained with a Unicam SP 200 spectrometer, taking care that the plane of the specimen was accurately located in the image plane of the spectrometer entrance slit; the specimen was then adjusted for maximum absorption. From the spectrum the deuterium content was obtained from the relative strengths of the NH and ND stretching bands *ca.* 3300 and 2400 cm^{-1} respectively, in the following way. As prepared the specimens were non-uniform; this causes the relative strengths of the various bands to depend in a complex manner on the precise distribution in extension and thickness of the part of the specimen imaged on the spectrometer slit. In addition, the area so imaged changes with wavelength, as a result of the slit closure mechanism. Since, however, certain bands in the spectrum are almost completely unaffected by the peptide deuterium content, it is possible to use these as controls to apply an approximate correction for the non-uniformity of the specimen. It is assumed that the specimen approximates to a

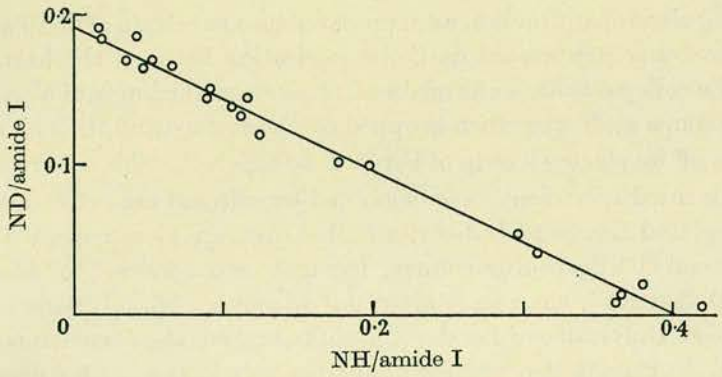

FIGURE 1. Graph showing the relation between the peak optical density of the NH and ND stretching bands, measured as a fraction of the amide I density, for a series of partially deuterated specimens of poly-γ-ethyl-L-glutamate.

uniform specimen which obstructs only a part of the beam, the remainder being transparent. A corrected 100 % absorption is then found to give the two control bands their correct relative strengths (which can be determined approximately in separate experiments) and this new 100 % mark then used to obtain the optical density of the NH and ND bands. Since the density of these two bands was never high and consequently not very sensitive to non-uniformities, the application of this approximate correction enabled reasonably accurate measurements to be obtained. In the case of the glutamic acid esters, the controls used were the ester C=O band at *ca.* 1730 cm^{-1} and the amide I band at 1660 cm^{-1}. For the remaining polymers, except poly-D-alanine, the CH stretching band was used with the amide I band. For poly-D-alanine, no correction was applied (Malcolm 1965) owing to the weakness of the CH band; fortunately this polymer gave very uniform films.

The extinction coefficients of the NH and ND bands can be quite different. This is shown clearly in figure 1, where the corrected peak optical densities of the NH and

ND stretching bands divided by the density of the amide I band (which is independent of the deuterium content) are plotted, one against the other, for the series of partially deuterated specimens used to measure the deuterium exchange rates of *PEG*. It will be seen that the intensity of the ND band decreases linearly as that of the NH band increases, and from the slope of the graph, the NH absorption is approximately 2·2 times stronger. The percentage of peptide hydrogen is then calculated from

$$\%\,\mathrm{H} = \frac{(\mathrm{NH})}{(\mathrm{NH}) + 2{\cdot}2(\mathrm{ND})} \times 100\,\%,$$

when (NH) and (ND) are the peak optical densities of the NH and ND stretching bands in a given specimen. Analysis of the results for other polymers has produced similar linear graphs, with values from the slope ranging from 1·33 to 2·3. To neglect this point, and calculate the deuterium content directly from the relative strengths of the NH and ND bands, as has been done in the case of proteins (Haggis 1957), is in general incorrect and can give rise to large errors.

Electron diffraction

Specimens for electron diffraction were prepared on a substrate of distilled water, the monolayers being compressed until the separation between the barriers was about 1 cm. The collapsed film so formed had an average thickness of about 200 Å. Electron microscope grids were then dropped on the surface and the polymer film and grids lifted off by placing a strip of Perspex, to which the film adhered, on the surface. The air-dried specimens were examined by selected area electron diffraction in an Associated Electrical Industries E.M. 6 electron microscope, with a field 6 μm diameter and 50 kV working voltage. The instrument was set to 'Alinement' rather than 'Diffraction', since as pointed out to me by Miss J. Smart (Queen Elizabeth College, University of London), usefully higher magnification is thereby obtained. Dr G. H. Haggis also provided valuable help in the early stages of this work. Both condensers were fully defocused and it was found essential to keep to a minimum the time the specimen was exposed to the electron beam. Exposure times of about 20's were used with Ilford HPS plates. Calibration rings from magnesium oxide powder were obtained by condensing the powder from a burning magnesium ribbon on part of some specimens. This also provided a check on distortions within the instrument. In the results quoted the departure from true circularity of the diffraction rings was less than 1 %.

Measurement of angles of contact

To measure the angle of contact between the polymer and a liquid, the usual dipping plate technique was used. Uniform thin clear films of polymer were prepared by drying down solutions spread evenly on microscope slides. An illuminated slit was viewed by reflexion at the liquid surface, and the angle of contact determined from the angle of the slide when set to give an undistorted image of the slit right up to the polymer surface. When the angle was greater than 90° this technique was modified by using a cell with transparent sides, so that an image reflected from the polymer/water/air contact would be viewed from below the liquid level by total

internal reflexion. In most cases two values for the angle of contact were obtained, the first when the freshly prepared film was brought into contact with the water, and a second a few degrees lower, after the film had been dipped below the liquid surface. This second value was used in the calculations that follow since, whatever the cause of the difference, the value so obtained is most likely to be the one characteristic of the adhesion of a monolayer to the liquid.

Experimental results and discussion

The force/area curves

Measurements of the force/area relations have been made on a range of substrates, usually distilled water, 0·01 N hydrochloric acid, 0·05 M phosphate buffer pH 7·5 and 0·05 M bicarbonate buffer pH 10. Very little dependence of the force/area curves on pH was found. This is contrary to the situation reported by Cheesman & Davies (1954), in the case of poly-DL-leucine. While their results agree with figure 2 for

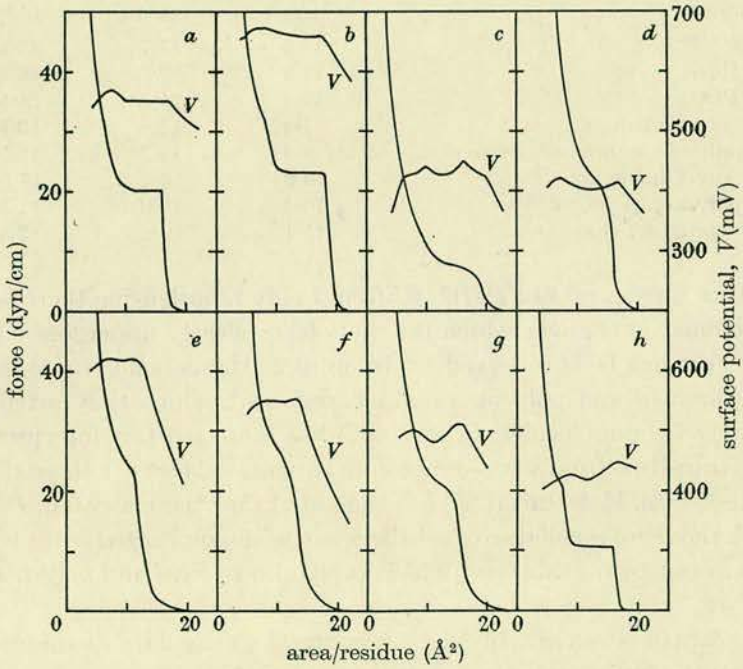

Figure 2. Force/area and surface potential (V) measurements for monolayers spread on hydrochloric acid pH 2, 20 °C. *a*, *PMG*; *b*, *PEG*; *c*, *PBG*; *d*, poly-DL-leucine; *e*, poly-D-alanine; *f*, poly-D-α-amino-*n*-butyric acid; *g*, poly-L-norvaline; *h*, poly-L-norleucine.

0·01 N hydrochloric acid, it has not been found possible to obtain agreement with the lower area they give for the polymer on 0·1 N sodium hydroxide. The possibility that the solvent they used might cause the polymer to be in a different conformation has been investigated by preparing monolayers from the solvent recommended by Davies (1953*a*), consisting of 80 % dichloracetic acid, 20 % isopropanol. This was found difficult to use, but with care the same area was found on both 0·01 N hydrochloric acid and 0·1 N sodium hydroxide. Since this solvent is more dense than water

and completely miscible with it, a low result might have been caused by polymer being carried with solvent into the substrate.

The area per residue, calculated by extrapolation of the first steep rise of the force/area curve to zero force, is compared in table 1 with the area calculated on the assumption that the molecules are in the α-helical conformation. The inter-helix distance used in this calculation is derived from the electron-diffraction observations (see below) on the assumption that there is hexagonal or near-hexagonal packing, and that the separation between helices in the monolayer is the same as in the specimens used for electron diffraction. It will be seen that there is reasonable agreement.

TABLE 1. COMPARISON OF MEASURED AREAS PER RESIDUE WITH ELECTRON DIFFRACTION OBSERVATIONS

polymer	(100) spacing (Å)	calculated area (Å²)	observed area (Å²)
PMG	10·2 (calc.)	17·7	17·5
PEG	11·3	19·7	19·6
PBG	12·5	21·6	21·5
poly-D-alanine	7·42	12·8	13·8
poly-D-α-amino-*n*-butyric acid	8·61	14·5	15·5
poly-L-norvaline	9·6	16·6	17·0
poly-L-norleucine	10·4	18·0	17·3
poly-DL-leucine	11·1	19·2	17·5

In the force/area curves of *PMG*, *PEG* and poly-L-norleucine there is a plateau at a well-defined force, over which the monolayer clearly undergoes a transition. A similar effect has been observed by Isemura & Hamaguchi (1952), in poly-DL-aminocaprylic acid and poly-DL-α-aminocapric acid, which they attributed to a reorientation of the molecules. Crisp (1958) has criticized this interpretation and suggested a transition from a two-dimensional orientated film to a three-dimensional disorientated state. Malcolm (1966) has suggested that the plateau in *PMG* is consistent with the regular collapse of α-helices in the monolayer to form a bilayer. The arguments in support of this view, which apply also to *PEG* and poly-L-norleucine, are as follows.

(*a*) The plateau is remarkably flat (in contrast to the data available to Crisp), which is a strong indication of a simple first-order phase change.

(*b*) The area per residue, at the end of the transition, is too low to accommodate any reasonable monolayer conformation.

(*c*) The experimental evidence both before the transition, from surface area and deuterium exchange measurements, and after, from infrared spectroscopy and electron diffraction studies, is consistent with the presence of α-helices.

(*d*) The surface potential remains fairly steady during the transition (see below).

(*e*) The proposed mechanism of bilayer formation gives reasonable quantitative data relating to intermolecular cohesive forces.

(*f*) If α-helices are present in the monolayer, they are in a condensed state and must therefore be packed in parallel groups. In addition, the temperature coefficient

of the pressure required to produce a bilayer is small and negative (in the range: $-0{\cdot}1$ to $-0{\cdot}2$ dyn cm^{-1} $°C^{-1}$). These facts suggest that both the initial and final states are ordered and that the entropy change in the transition is small.

In the remaining polymers in figure 2, there is an inflexion rather than a plateau, suggesting that a similar type of transition may be occurring in the other monolayers, but that either the initial or final state is in some way more disordered. In experiments still proceeding with poly-β-benzyl-L-aspartate, high molecular weight material has been found to give a plateau, whereas low molecular weight polymer (mol. wt. 5000) gives an inflexion at about the same pressure. With this polymer the length of the plateau depends to some extent on the spreading procedure used to form the monolayer, but with the group of polymers considered here, any such effect is slight. Attempts to prepare monolayers with a plateau from polymers giving an inflexion, have been made. Two methods were used, first to spread monolayers at ten times the normal dilution of chloroform, and secondly to spread on water at 45 °C and allow the temperature to fall to 20 °C before taking measurements. These approaches produced no detectable effect. Thus the shape of the force/area curve appears predominantly to be an intrinsic characteristic of the particular polymer specimen.

When the bilayer is more or less complete, under further compression the film must continue to increase in thickness either as a result of the regular formation of further layers, or folding, or both. If compression is stopped the film relaxes, and the force/area curve is therefore dynamic. Relaxation is, however, slow; any convenient rate of compression, with up to an hour for obtaining the curve, produces essentially the same form of curve up to 70 dyn cm^{-1} or more, when the film suddenly becomes unstable and collapses.

On the basis of the proposed transition from monolayer to bilayer, considerable information may be derived concerning the cohesive forces involved. The work required to remove unit area of monolayer from the water surface $W_{P/L}$, can be calculated from Young's equation, assuming that this equation (Elliott & Riddiford 1964) derived for a smooth solid, may be applied to a monolayer:

$$W_{P/L} = \gamma_{L/A}(1 + \cos\theta).$$

$\gamma_{L/A}$ is the surface tension of the liquid with respect to air and θ the angle of contact of the polymer/liquid interface. The work W done on the film during compression to form unit area of bilayer is numerically equal to the pressure required to form the plateau, plus a small correction for the energy stored in the compressed bilayer amounting to not more than about one erg cm^{-2}. The values calculated for the work of adhesion $W_{P/L}$, are greatly in excess of the values obtained for W, showing that the principal source of energy for the transition from monolayer to bilayer is the free energy of the polymer/vapour interface, $F_{P/V}$. Thus the removal of unit area of monolayer from the water surface to form a bilayer may be expressed by the equation:

$$2F_{P/V} = -\gamma_{L/A}(1 + \cos\theta) + W.$$

This assumes that the polymer is sufficiently hydrophobic for the work required to displace any adsorbed water molecules from between the two layers of polymer as

the bilayer forms to be negligible. If this is so, $2F_{P/V}$ is approximately the work of cohesion of the polymer, since it is reasonable to assume that the work of cohesion between two monolayers is the same as between similar surfaces in a thick specimen (but see below).

Table 2 shows the values calculated from data for monolayers on distilled water. The values for θ are not very accurate (approximately $\pm 2°$) and it has therefore not been thought justifiable to apply small corrections for the energy stored in the compressed bilayer. Since the inflexion observed when a plateau is absent appears to have the same general characteristics and to arise in the same general way as the plateau, its position has been used to provide a value for W, albeit rather less accurately; it is not essential for the theory that the bilayer formation shall proceed to completion, all that is assumed is that the plateau or inflexion represents the region where molecules leave the water surface and start to form a second layer in a regular manner.

TABLE 2. APPLICATION OF YOUNG'S EQUATION TO CALCULATE ENERGIES OF COHESION (20 °C)

polymer	θ (deg)	pressure to form bilayer (dyn cm^{-1})	$W_{P/L}$ (erg cm^{-2})	$2F_{P/V}$ (erg cm^{-2})	$2F_{P/V}$ (kcal/mole pair residues)
PMG	58	20	111	91	3·43
PEG	63	22	106	84	3·56
PBG	73	6†	94	88	4·09
poly-D-alanine	42	25†	127	102	3·04
poly-D-α-amino-*n*-butyric acid	57	25†	112·5	87·5	2·9
poly-L-norvaline	87	20†	76·6	56·6	2·1
poly-L-norleucine	94	10·5	67·8	57·3	2·1
poly-DL-leucine	97	20†	63·8	43·8	1·6

† Approximate value.

To express $2F_{PV}$ on a molar basis and attribute it to the interaction of the side-chains between the planes, it is necessary to make some assumptions concerning the packing of the second layer of molecules on top of the first. In the absence of any evidence to the contrary, it will be assumed that the helices pack in the normal hexagonal manner and that, in an infinite structure, cohesion between (100) planes can be attributed to two-thirds of the total number of residues. The values of the cohesive energy so obtained expressed on a molar basis, are mean values for the many ways in which the side-chains can pack together in a hexagonal structure (see, for example, Elliott & Malcolm 1959).

The pressure required to form a bilayer should decrease as the work of adhesion decreases and this has been investigated for *PMG* and *PEG*. Figure 3 shows a graph of W against $W_{P/L}$ for *PEG* monolayers on water/isopropanol mixtures in the range of 0 to 4 % isopropanol giving a range of $\gamma_{L/A}$ from 72·8 dyn cm^{-1} to 54 dyn cm^{-1} and of θ from 63 to 53°. If $F_{P/V}$ were independent of the liquid then from the equations above, the graph should be linear with unit slope. The observed variation of W with $W_{P/L}$ is good support for the validity of the overall picture, but the slope is not so

steep as would be expected if $F_{P/V}$ were independent of the composition of the substrate. Similar results have been obtained using water/acetone mixtures, and it has been found for both liquid mixtures that a graph of W against $\gamma_{L/A}$ is linear with a slope of approximately unity. If the correct value of θ were around 90° and the observed value too low, this would account for the result, without postulating a dependence of $F_{P/V}$ on the composition of the substrate. This appears unlikely since for polyethylene θ is only 93° and *PEG* would be expected to be considerably less hydrophobic, and hence with a correspondingly lower value for θ. It must therefore be concluded that $F_{P/V}$ depends to some extent on the composition of the substrate. This may be caused by either a direct interaction between the substrate and the film, or indirectly as a result of adsorbed vapour molecules influencing the formation of the bilayer. Clearly this should be the subject of a further investigation.

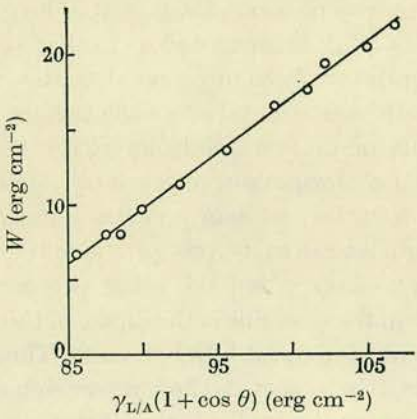

FIGURE 3. Graph showing the relationship between the work W required to form a bilayer and the work of adhesion of the polymer to the liquid, for *PEG* on water-isopropanol mixtures 20 °C.

The validity of the foregoing also rests on the use of Young's equation which, as has been pointed out by Lester (1961), may not be valid for polymers, since the component of the liquid surface tension normal to the solid surface may cause it to deform and produce an error in θ. It is suggested that this may be important if the Young modulus for the solid is less than 10^{10} dyn cm^{-2}. An approximate value for *PEG*, obtained by measuring the deformation of a thick undrawn fibre loaded at one end, is 2×10^9 dyn cm^{-2}; there may therefore be an error in the value of θ. However, the thickness of the surface layer of water, assumed to cause the deformation, is perhaps 10 Å, comparable with the diameter of an α-helix. Therefore, in contrast to rubbers and similar materials considered by Lester, where individual molecules are probably flexible, Young's modulus for *PEG* may not be related very closely to the deformability of small areas of specimen composed of α-helices, possibly in a para-crystalline array. These considerations lead on to a realization that even if any deformation is slight, the surface may not be 'smooth' as is assumed in the derivation of Young's equation. The effect of deformation and irregularities would probably be to make the true values of θ higher.

The values in table 2 must therefore be considered inexact. Nevertheless, they

appear consistent and reasonable and this is good support for the interpretation of the plateau and inflexion in the force/area curves. Moreover, in the absence of more precise methods, the results indicate a new experimental approach to understanding molecular cohesion in polypeptides and perhaps other polymer systems. The data is of particular interest in relation to understanding protein/water and protein/protein interactions.

Surface potential measurements

The dependence of the surface potential on area (figure 2) can be understood in terms of the picture derived from the force/area curves. At areas well above the condensed area, the surface potential varies widely across the surface, showing that the monolayer is condensed into large-scale aggregates. As the monolayer is compressed so that it begins to exert a pressure, the potential becomes uniform over the surface and rises steadily to a peak at an area close to that at which the transition starts. This monolayer potential has been interpreted, in the case of poly-D-alanine as arising from a change in the net orientation of the surface water molecules consequent upon spreading the monolayer (Malcolm 1965); this must be so if the molecules are in the α-helical conformation, since a long α-helix, which has a nearly symmetrical array of dipoles in the backbone and the side-chains, can have almost no net dipole moment at right angles to its axis, provided it remains undistorted at the water surface. In poly-D-alanine and the other polymers with hydrocarbon side-chains, the main dipole in the molecule is the dipole of the peptide group, which is quite polar with a moment of about 3·7 Debye units. Thus dipolar interactions with the substrate water are to be expected; the positive sign of the potential shows that the positive end of the water dipoles tends to orientate towards the monolayer, with perhaps a weak hydrogen bond between a water hydrogen and the carbonyl group. A more complicated situation arises with the esters of poly-L-glutamic acid, since any distortion of the side-chains at the interface will destroy the helical symmetry and in general give rise to a net dipole moment. In addition the side-chain dipoles, as well as the peptide groups, will interact with the underlying water. It is possible that the net effects of the side-chains on the potential is small, since the observed potentials are comparable to those of the other polymers.

For the polymers with normal hydrocarbon side-chains, the initial rise in the surface potential is proportional to the increase in the number of residues per unit area. This is probably a consequence of the condensed aggregates becoming more closely packed and to some extent, when the side-chain is long, as a result of the side-chains being distorted under pressure. The surface potential per residue, at the end of the initial rise, is approximately the same for all four polymers. This suggests that the interaction of the peptide group with the water is independent of the side-chain size, and that any ordering of the water around the side-chains on the underside of the helices does not affect the surface potential.

When a bilayer starts to form, no change in the surface potential is to be expected, provided the helices in the second layer are undistorted and do not interact with either the substrate water or with any adsorbed water molecules present in the film. It will be seen that for both *PEG* and *PMG*, where there are well-defined plateaux

in the force/area curves, there are corresponding plateaux in the surface potential. This is not the case with poly-L-norleucine which, like most of the remaining polymers, shows a small decrease in the surface potential when the bilayer starts to form. If in these instances the first molecules to move into the upper layer are those which occupy the smallest area per residue, and we assume that the dipolar contribution of the water depends only on the number of residues per unit area in the lower layer, loss of the most closely packed molecules to the upper layer would account for the observed decrease. This would be expected to occur in the most crystalline regions of the specimen. As the bilayer continued to form compression of the remaining less crystalline areas of monolayer would then cause the potential to rise again. A contributory factor here is probably the compressibility of the molecules. While the backbone of the helix is relatively rigid, long side-chains would be expected to show deformations under pressure and it is therefore of interest to note that during the formation of a bilayer the fluctuations in the surface potential are most marked in the polymers with long side-chains.

An important general conclusion from the surface potential measurements is that the net reorientation of the water molecules induced by spreading the monolayer can give rise to large potentials. The usual practice of combining the unknown contribution of the water molecules with the monolayer dipoles and expressing the result as a moment per monolayer molecule (Adamson 1960; Davies & Rideal 1961) has therefore little to commend it. There is then the tendency to interpret the dipole moment directly in terms of the conformation of the monolayer, as has been done by Davies (1953*a*, *b*) for poly-DL-leucine and other polypeptides with, on the evidence presented here for poly-DL-leucine, incorrect conclusions.

The deuterium exchange measurements

The deuterium exchange curves (figures 4 and 5) show that the technique described produces consistent results. No correction has been applied for any initial exchange during spreading of the monolayer or for the possibility that the polymer was not quite 100 % deuterated. Each exchange experiment was timed from the moment of completion of spreading the monolayer, so that these quantities are included in the extrapolation to zero time on the exchange curves. So far as possible the procedures used were standardized, nevertheless variations were observed, which may be attributed to effects caused by differing rates of removal and drying of the film, and high accuracy is not claimed for the experimental data. However, interpretation of the results depends only on the general pattern of the exchange reaction and this is well established by the results. For the polymers considered here, of high molecular weight, there is no observable dependence of the rate of exchange on the area available to the monolayer and compression of the monolayers to 5 dyn cm^{-1} did not appear to affect the results.

It is reasonable to neglect any reverse reaction and to analyse the results as a series of one or more first-order reactions. If f is the fraction of peptide groups which have not exchanged at a time t then

$$1-f = \sum_i f_i e^{-k_i t}, \quad \sum_i f_i = 1,$$

when f_i is the fraction of the total number of peptide groups with a first-order rate constant k_i for i different types of sites. In no case, making generous allowance for experimental errors, is it possible to fit any of the exchange curves using only one exponential term. In general two or three terms are necessary at each value of pH.

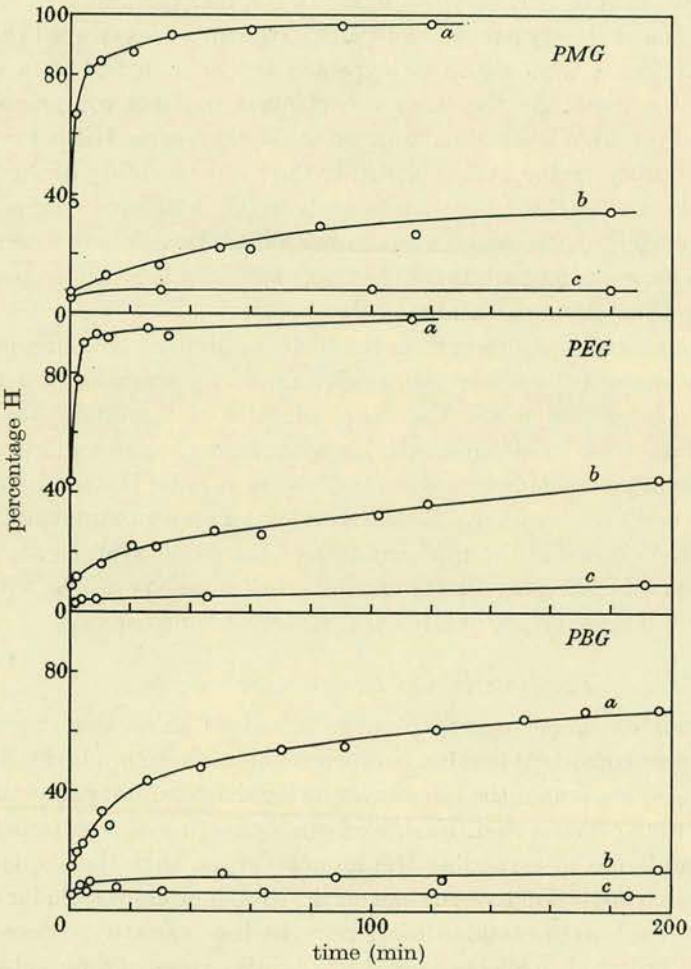

FIGURE 4. Deuterium/hydrogen exchange curves for monolayers on substrates of different values of pH. (*a*) 0·05 M bicarbonate buffer pH 10, (*b*) 0·05 M phosphate buffer pH 7·5, (*c*) hydrochloric acid pH 2. Upper graphs *PMG*; middle graphs *PEG*; lower graphs *PBG*.

A few experiments were continued for periods up to 24 h and these suggested that an additional term was necessary to account for the final very slow exchange. This has not been given separately in the analysis. Experiments over longer periods would be desirable; however, occasional darkening of the films and the possibility of de-esterification under alkaline conditions of the esters of polyglutamic acid, suggested that experiments over long periods might be suspect.

Table 3 gives approximate rate constants derived from the results given in figures 4 and 5 and the data for poly-D-alanine (Malcolm 1965), at pH 7·5 and pH 10.

Fractions with an exchange half-time of less than 1 min have not been included in the table. At pH 2 the exchange was too slow to obtain reasonably accurate rate constants, though there appeared always to be a small initial exchange followed by a very slow reaction.

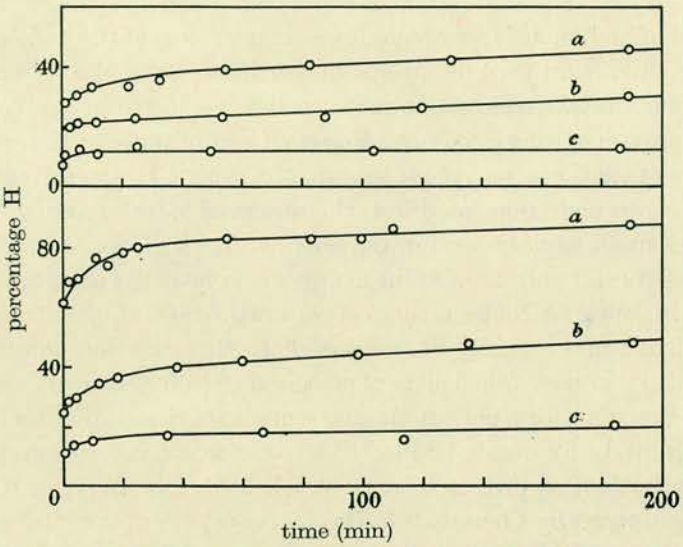

FIGURE 5. Deuterium/hydrogen exchange curves for monolayers on substrates of different values of pH. (*a*) 0·05 M bicarbonate buffer pH 10, (*b*) 0·05 M phosphate buffer pH 7·5, (*c*) hydrochloric acid pH 2. Upper graphs poly-L-norvaline; lower graphs poly-DL-leucine.

TABLE 3. RATE CONSTANTS FOR DEUTERIUM EXCHANGE IN MONOLAYERS, 20 °C FOR FRACTIONS WITH $t_{\frac{1}{2}} > 1$ MIN

polymer	pH	f (%)	k (min^{-1})
PMG	7·5	23	0·02
		70	5×10^{-4}
	10·0	43	0·23
		10	0·07
		10	0·01
PEG	7·5	12	0·2
		80	0·002
	10·0	50	0·2
		5	0·08
PBG	10·0	34	0·047
		54	0·0026
poly-D-alanine (22 °C)	7·5	5	0·2
		10	0·014
		60	7×10^{-5}
	10	20	0·2
		10	0·08
poly-L-norvaline	10	15	0·07
		60	0·0043
poly-DL-leucine	7·5	16	0·09
		60	0·0023
	10	20	0·09
		20	0·0023

It has not proved possible to measure the activation energy for the exchange reactions since, in experiments to test this possibility, it appeared that not only were the rate constants k_i a function of temperature but also the coefficients f_i. This is in contrast to the situation found in insulin by Hvidt & Linderstrøm-Lang (1955).

For material of high molecular weight, exchange at sites at the ends of individual molecules (e.g. unbonded peptide groups in α-helices) can make only a very small contribution to the total exchange, so that it will be neglected in analysis of table 3. These results provide strong evidence against all conformations hitherto proposed for monolayers of synthetic polypeptides except the α-helix (or related helical conformations) for two main reasons. First, the observed rates are much slower than are observed in small peptides and in polymers where hydrogen bonding to water can take place. Thus for poly-DL-alanine in aqueous solution at pH 7·5, the empirical formula given by Bryan & Nielsen (1960) gives a rate constant of 150 min^{-1} at 20 °C, much faster than could actually be measured. In this case the polymer is either hydrogen-bonded to water, or in helices of marginal stability. The difference between this value and the value for a poly-D-alanine monolayer is too great for the polymer in the monolayer to be hydrogen-bonded to water. Secondly, conformations similar to the β-conformation, as proposed by Mishuck & Eirich (1955) or the extended conformation proposed by Cheesman & Davies (1954) are unacceptable since both these have all their peptide groups similarly situated with respect to the interface, and there should therefore be only one rate-constant for the exchange reaction. Even if allowance is made for local variations in the environment of some of the peptide groups, the wide range of the observed rate constants in monolayers can hardly be accounted for in this way.

The proposal that the α-helix is the stable conformation in the monolayer (Malcolm 1962, 1965) can form the basis of a satisfactory explanation of the deuterium exchange data. Since it is a structure with intra-molecular hydrogen bonds the general slowness of the reaction is accounted for. In addition, it is to be expected that at a given pH the rate of exchange of a group will depend markedly on whether it is on the underside of the helix, between helices or exposed to the vapour. While peptide groups most closely in contact with the substrate will clearly be the first to exchange, it is not perhaps wise to try to identify at this stage any particular rate constant at a given pH with a particular group of exchangeable sites.

Distortion of the helix arising from the asymmetrical environment cannot be ruled out, nor do these experiments exclude other helical conformations. One assumption implicit in this explanation is that the molecules do not rotate appreciably about their axes over the duration of an experiment; if they did, then on a time-average all the peptide groups would be exposed to the same exchange conditions and there would therefore be only one rate-constant. The cohesive forces between the molecules and interpenetration of the side-chains would be expected to prevent any rotation, nevertheless a slow random rotation within a monolayer aggregate by a cog-wheel action is perhaps conceivable, and it is therefore satisfactory to have evidence that this is not appreciable.

The deuterium exchange reaction

The exchange experiments, along with the force/area and surface potential measurements, lead to a consistent and simple picture of the polypeptide monolayer. The molecules appear to be in a helical conformation, which in the absence of any evidence to the contrary will be assumed to be the α-helix; indirect observations, from infra-red spectroscopy and electron diffraction (see below) strongly support this assumption. Since helices are essentially rod-like, the cohesive forces then cause the molecules to condense and form parallel arrays with a high degree of order, as is shown in some instances by the plateaux in the force/area curves. The monolayer thus appears to be essentially a solid, with the individual molecules having little freedom to rotate, or to flex within or out of the plane of the interface. Such a structure is of value as a model system for understanding reactions of biological interest, and one immediate application is to a better understanding of the deuterium exchange reactions used to study protein structure. It must be concluded from the stability and regularity of the monolayer that, while some exchange may first take place around the edges of monolayer aggregates and at the ends of helices, the bulk of the observed exchange arises from a reaction in which the helices are not allowed to 'break' or flex. The reaction mechanism must be one in which the deuterium is removed from the helix with little change in the coordinates of the other atoms of the peptide group. That exchange at a sufficiently high pH might take place by such a process, has not previously been shown. Previous experimental investigations on synthetic polypeptides have been largely restricted, for solubility reasons, to poly-L-glutamic acid in aqueous solution (Blout, de Lozé & Asadourian 1961) in which very slow exchange was observed at pD 3·5. Under less acid conditions, where exchange was faster, the α-helix was becoming less stable and conformational changes were probably exposing intramolecularly bonded peptide hydrogen atoms to solvent (Hvidt & Nielsen 1966).

Thus it has been supposed that the exchange rate of an intact α-helix that is not allowed to 'break' in order for exchange to proceed might be expected to be negligible under all conditions normally encountered in protein hydrogen exchange work. On this basis Hvidt & Nielsen (1966) have presented a detailed analysis of deuterium exchange in proteins. It is assumed that in any protein molecules in which the macromolecular conformation is hypothetically considered to be fixed, practically all the peptide group hydrogen atoms fall into two classes of labile hydrogen atoms, one in which the exchange rate is immeasurably slow, and one which is rapid, approaching that of low molecular weight polypeptides. The fraction that is observed to exchange slowly is then attributed to reactions arising from a transconformational change in the protein molecule.

It would appear, from the exchange data on monolayers, that the postulate of a transconformational change is not always necessary if α-helices are present in the protein, since a range of slow exchange rates would be expected in helical regions, determined by the pH and the accessibility of the peptide groups to water. In fact, a monolayer of α-helices can be considered as a model of a perfectly stable protein, composed entirely of helices, in which the monolayer/water interface represents the

outer surface of the protein and the monolayer/vapour interface the interior. This model shows that even in the absence of a conformational change, exchange would be expected except under certain acid conditions. Thus the deuterium exchange measurements of Jordan & Speakman (1967) on wool, at an arbitrary pH, are not a reliable direct measurement of α-helical content; the agreement they obtain with other methods is probably a consequence of the fact that the α-helical regions in wool are probably the most crystalline and resistant to penetration by water.

It must be concluded therefore that under conditions of pH and temperature where an intact helix can exchange, the reaction scheme proposed by Hvidt & Nielsen may require modification, but it is probably satisfactory at pH 2 to 3 where intact helices exchange very slowly.

The deuterium exchange curves and the data in table 3 give some further qualitative evidence concerning the effect of the hydrophobic character of the side-chain on the exchange rate. Thus *PBG* exchanges much more slowly than *PMG* or *PEG* at the same pH; similarly, exchange in poly-L-norvaline is much slower than in poly-D-alanine. Poly-DL-leucine is exceptional, almost certainly because with equal numbers of D and L residues it is composed of either short lengths of right- and left-handed helices with intervening breaks, or of helices with both L and D residues in them, which in either case will be a less stable structure than a helix composed entirely of one enantiomorph. A consequence of this is that the initial exchange is larger than in the other polymers.

Although *PEG* is more hydrophobic than *PMG* it appears to exchange slightly faster. There may be several reasons for this. The electron diffraction patterns from the two polymers (see below) suggest that a *PEG* monolayer may be less crystalline than *PMG*, so that it might be more penetrable to water and hence exchange more rapidly. This may be helped by the additional methylene group which, while increasing the hydrophobic character of the polymer, also increases the separation between the helices. Moreover, a hydrophobic group at the end of a side-chain may have less effect on the exchange than when it is close to the peptide group. It will also be noted that the surface potential of a compressed monolayer of *PEG* is exceptionally high, suggesting again a particularly strong interaction with water.

Finally it should be remembered that, since the exchange depends on the pH of the substrate, a full discussion of the exchange mechanism will involve the question of the accessibility of the peptide groups to solute as well as to water.

The molecular structure of the collapsed monolayer

When monolayers were collapsed and removed from the surface, their infrared spectra were in all cases consistent with the α-helical conformation. This applied to both the normal and the deuterated specimens. If therefore any of the polymers had been in the β-conformation at the interface, it is necessary to postulate a complete conformational change caused by removing the monolayer, since the β-conformation can be recognized from the position of the amide I band. In early work, however, spectra showing signs of the β-conformation were obtained from monolayers of *PMG* spread on alkaline substrates (Malcolm 1962). This was correlated with a force/area curve that was more hyperbolic than usual and deuterium exchange

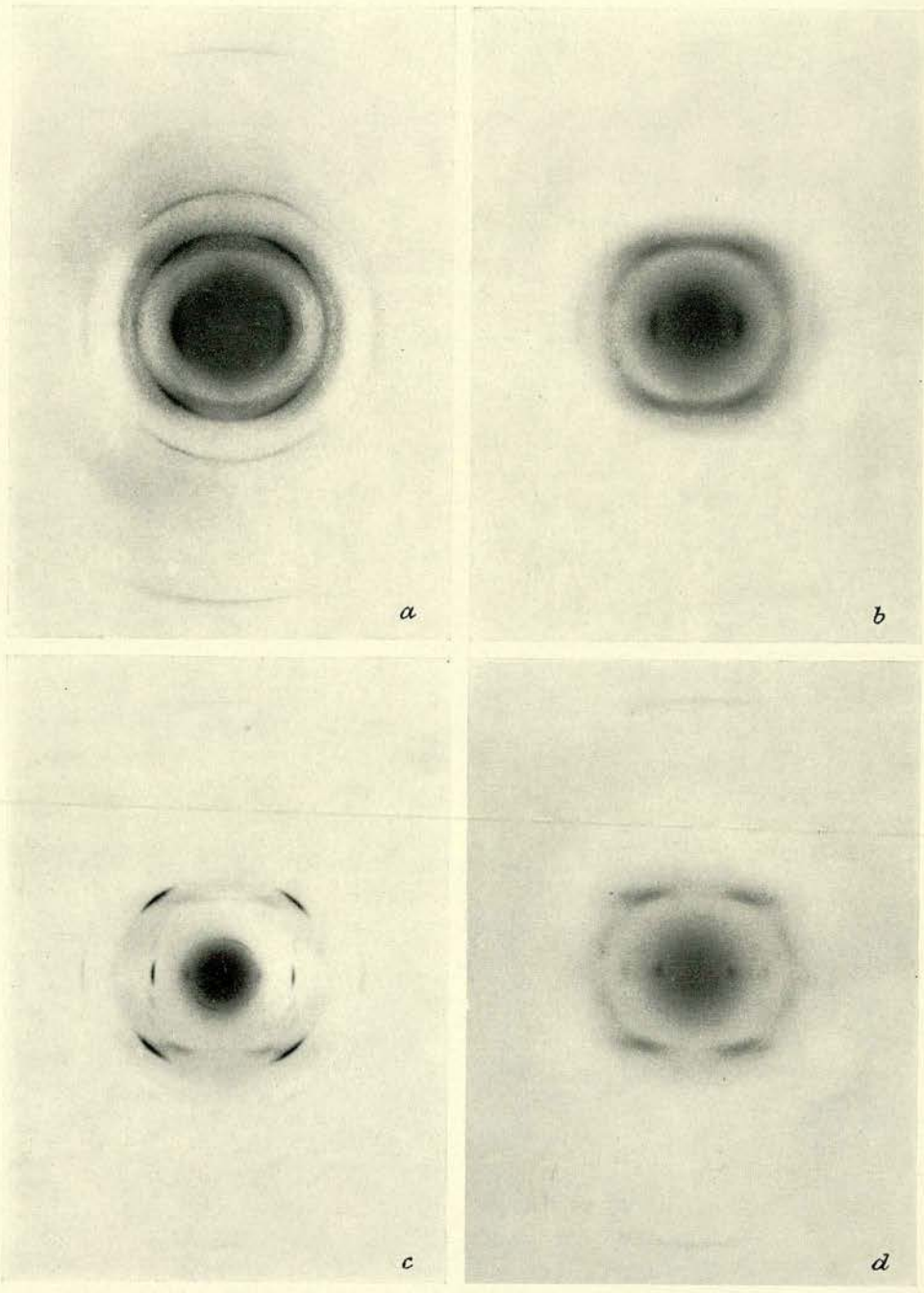

FIGURE 6. Electron diffraction patterns from collapsed monolayers spread on distilled water. (Magn. × 2.) In all cases the outermost reflexion corresponds to a spacing of 1·495 ± 0·005 Å: (*a*) poly-D-alanine, (*b*) poly-L-norvaline, (*c*) *PMG*, (*d*) *PEG*.

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which depended on the area per molecule. It appears now that the material used for this early work was of low molecular weight, possibly as a result of degradation during storage, and these results need repeating with well characterized low molecular weight material. Fresh high molecular weight material gives the type of behaviour described in this paper.

Additional information has been obtained by electron diffraction examination of the collapsed monolayers, and the photographs in figure 6, plate 4, are typical of the patterns obtained. In all cases the observation of a meridional reflexion at 1·5 Å and, with exceptions to be discussed, a general correspondence of the positions and intensities of other reflexions with those obtained in X-ray diffraction studies of specimens shown to be in the α-helical conformation, show that after removal of the collapsed monolayers from the surface the conformation is that of the α-helix. It is clear also that the method of forming the specimen causes the development of a high degree of orientation, and the molecular axes have been found to orientate parallel to the

TABLE 4. OBSERVED AND CALCULATED SPACINGS FOR *PMG* FOR A HEXAGONAL CELL, $a = 11{\cdot}80$ Å, $c = 27$ Å

d (obs.) (Å)	d (calc.) (Å)	h k l
absent	10·22	1 0 0
5·89	5·90	1 1 0
2·95	2·95	2 2 0
5·44	5·40	1 1 2
4·88	4·93	1 1 3
4·01	3·98	1 1 5
2·93	2·93	1 1 8
1·50	1·50	0 0 18

barriers used to collapse the film. This is to be expected if groups of parallel molecules in no particular orientation, collapse to form anisotropic structures. Viscous drag during compression would then be expected to cause orientation with the long axes at right angles to the direction of compression. This appears to be a new method of preparing thin, highly oriented, polymer specimens. In the case of poly-D-alanine, deliberate stretching of the film not only improved the orientation but also produced additional reflexions which could be indexed on the cell proposed for the β-conformation (Brown & Trotter 1956). It is therefore emphasized that care has been taken to avoid orientation caused by stretching the specimen during its removal from the surface.

In general there is not a sufficient number of reflexions to warrant a detailed investigation of the unit cells, and table 1 gives the (100) spacings calculated assuming hexagonal packing. The spacing for poly-D-alanine agrees within the limits of experimental error with the value given by Brown & Trotter (1956). The spacing of 12·5 Å for *PBG* may be compared with 12·6 and 13·2 Å given by Bamford *et al.* (1956) in a specimen showing double orientation. *PEG* gives equatorial reflexions at 11·3 and 5·75 Å (figure 6*d*) compared with the value of 12·03 Å from X-ray studies on material prepared as film or thick fibres (A. Elliott & L. Brown, and M. Harding, private communications). These differences are probably real and a consequence of the ways the specimens are prepared.

In contrast to *PEG* and *PBG*, *PMG* is particularly crystalline (figure 6*c*) and a number of reflexions have been indexed on a cell close to that proposed by Bamford, Brown, Elliott, Hanby & Trotter (1952) (table 4). There are, however, far fewer reflexions than in the usual fibre photographs of this polymer, and this can be understood if the specimen approximates to a single crystal with the *a*- and *c*-axes perpendicular to the ~~film~~. The generally complete absence of the strong 100 reflexion that dominates fibre photographs is particularly striking. Only occasionally have weak 100 and 210 reflexions been observed, showing that usually the *a*–*c* planes are not inclined at more than 10° to the plane of the specimen. In some specimens a weak equatorial arc *ca.* 11·4 Å has been observed, and sometimes in addition a longer sharp arc at the same spacing but not symmetrical with respect to the rest of the diagram, as can be seen in figure 6*c*. The origin of these reflexions remains obscure, but one possibility is that they arise as a first-order reflexion from a simple monolayer lattice of parallel α-helices. Finally it will be seen that there is a strong layer line streak on the fifth layer line which, as a consequence of the few crystallographic reflexions, is clearly observed. This probably has its origin in a measure of randomness in the antiparallel arrangement of the molecules, as has been found for poly-L-alanine (Elliott & Malcolm 1959).

All the polymers with hydrocarbon side-chains except poly-D-alanine generally show poor crystallinity and give diffraction patterns similar to poly-L-norvaline (figure 6*b*). It is of interest to note, however, that the densities of the polymers with normal hydrocarbon side-chains calculated from the electron diffraction data assuming a hexagonal cell, show a progressive decrease: 1·24, 1·10, 1·04 and 1·00 g/ml as the side-chain increases from one to four carbon atoms. Since the backbone of the helix must remain of almost constant density, this large decrease must arise from the increasingly poor packing of the side-chains. Thus while poly-D-alanine packs exceedingly well (Elliott & Malcolm 1959) and gives a high cohesive energy, the helical arrays of longer normal hydrocarbon side-chains are not fitted to dense packing and consequently the cohesive energies (table 2) show a corresponding decrease. The density calculated for poly-DL-leucine, 0·88 g/ml. is also very low, as is the cohesive energy. These correlations are good indirect support for the general validity of the interpretation of the significance of the force/area curves and the application of Young's equation.

The mechanism of bilayer formation

In the light of all the experimental results it is possible to put forward a tentative model for the way in which a bilayer forms. It is likely that when the monolayer is under pressure, a number of individual molecules are forced out of the lower layer and act as nuclei for the formation of the second layer. Once this has happened, the second layer will be formed as a cooperative process as shown in figure 7. Of the two molecules *A* and *B* in the lower layer, *B* is to some extent more stable than *A*, owing to the packing of *C* above. If there is an interaction between *A* and *C* through attractive forces between side-chains, then *A* will be the least stable molecule in the structure and forced into the upper layer. This process will then be repeated, the

work per residue required to move each molecule to the upper layer remaining constant.

This process will occur most readily if the side-chains are sufficiently long to interact, or where there are dipoles in the side-chains which will, if they are sufficiently close, produce an attractive force as in *PMG* and *PEG*; for *PBG*, however, dispersion forces between benzyl groups may be more important. This scheme also explains qualitatively why, in the other polymers, four carbon atoms are necessary in the side-chain before a plateau appears, though it should be realized that, assuming reasonable inter-helix distances, a four-carbon side-chain is not sufficiently long for the side-chains of molecules *A* and *C* to be in contact. Spontaneous thermal fluctuations are probably important in all cases in initiating a movement and these will be most marked when intermolecular cohesion in the monolayer is weak. This could be another reason why poly-L-norleucine shows a flat plateau whereas poly-D-alanine does not.

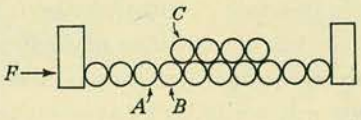

FIGURE 7. The collapse of a polypeptide monolayer to form a bilayer, molecules viewed end on.

When only an inflexion is observed in the force/area curve, the formation of the second layer probably proceeds less smoothly and produces a less regular structure. The energy differences involved over the length of an inflexion are, however, very small; an increase of 1 dyn cm^{-1} in the pressure required to force a molecule out of the surface, corresponds to only 0·03 kcal/residue at 20 $Å^2$/residue.

When the bilayer is complete, it is possible that a third layer may in some cases form by a similar process, though in this case two molecules must be moved, one into the third layer and one into the second. There is some evidence for this, the force/area curves of both *PMG* and poly-L-norleucine both show definite changes of slope around 6 $Å^2$/residue; in addition, the 'single crystal' diffraction pattern of *PMG* shows clearly that the final structure has the same orientation of the crystallites throughout the thickness of the film. This last point is not entirely conclusive since it is difficult to rule out recrystallization at a later stage in the collapse of the specimen. The diffraction pattern of poly-D-alanine also shows that at some stage a regular structure must form over a thickness sufficient to give a well-defined hexagonal pattern. Certainly the structures develop considerable strength and before final collapse will sustain, during slow compression, a pressure of 70 dyn cm^{-1}.

Some of these points concerning the formation of multilayers might be answered by direct electron microscopy of the specimens at high power. At low power and in the light microscope some films, notably *PEG*, looked remarkably uniform and were almost invisible except for regions where the film had clearly folded in the last stages of collapse. Other films had a characteristic texture which it would be of considerable interest to examine under high power.

Conclusions

A reasonably satisfactory picture of the structure and properties of these monolayers can clearly be developed. In the past, undue reliance has been placed on the application of the standard and often not very precise methods of surface chemistry, without attempting to check the conclusions against those drawn from other fields of structural investigation. The value of the use of a wide range of techniques is clearly shown, particularly when these are applied to a series of related polymers. While at the outset of this work there were reasonable indications, both experimental and theoretical, that the α-helix was probably stable in these polymers at the air/water interface, the search for experimental proof has produced a number of new pieces of information which, though in retrospect they might have been expected, have given additional interest to the work.

There is now good evidence that in monolayers of the polymers we have considered, the α-helix is stable at the air/water interface, and that the molecules pack in a regular array with little freedom of rotation about their axes. This conclusion has an important bearing on the significance of surface potential measurements, both in relation to these polymers and in surface chemistry generally. The deuterium exchange measurements not only contribute to the evidence for the structure of the monolayer but show also the conditions under which exchange can take place in an α-helix, the effects of the hydrophobic side-chains and of the accessibility of the peptide groups to water.

Perhaps the most interesting new result is the flat plateau observed in some of the force/area curves, which is remarkable for a transition in a polymeric system. This is being further investigated and it is possible that it may be observed in other types of polymers. While the explanation of the plateau appears reasonable, the conditions under which it forms and its shape, need further experiments before a full understanding is reached.

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Right-handed α-Helix and Conformational Changes in Poly-β-benzyl-L-aspartate

THE need to use non-aqueous solvents has limited the biological value of experimental work on many synthetic polypeptides. Considerable interest has therefore been shown in their surface chemistry, because, quite apart from the importance of understanding the conformation of polypeptides at interfaces, the properties of the polymer when exposed to water can also be investigated. From a combination of the classical methods of surface chemistry with the normal methods of structural investigation, there is at present good evidence to show that the α-helix is present in molecular monolayers of a number of synthetic polypeptides at the air/water interface[1]. This work has been extended to poly-β-benzyl-L-aspartate, and in this communication I consider chiefly the properties of the polymer after removal from the water surface; full details of the surface chemistry will be given elsewhere. In contrast to most synthetic polypeptides composed of L-residues, this polymer normally exists in solution and in the solid state as a left-handed rather than a right-handed α-helix[2,3]. If a specimen is heated to 160° C it undergoes a transition to the ω-helix, containing four residues per turn, which also appears to be left-handed[4]. Orientated specimens have characteristic infra-red absorption frequencies and dichroism enabling the two conformations to be clearly recognized and distinguished from the right-handed α-helix[4].

I have examined two polymer specimens, with molecular weights given as 250,000 and 130,000, from Pilot Chemicals Incorporated and the Sigma Chemical Company, respectively. In all respects the results from the two specimens were very similar. Monolayers were spread on distilled water in a Langmuir trough using 0·05 ml. of solution containing about 1 mg/ml. of polymer, the solvent being either chloroform containing 1 per cent dichloroacetic acid (in which the polymer is α-helical) or chloroform with 10 per cent dichloroacetic acid (in which the polymer is randomly coiled[2]). From either solvent the force/area curve of the monolayer gave a condensed area of about 20·5 $Å^2$/residue (consistent with a monolayer of α-helices) and a plateau at a pressure of 10 dyne cm^{-1} which, as in certain other cases[1], probably arises from the regular collapse of the α-helices in the monolayer to form a bilayer.

As with other synthetic polypeptides, it has been found that if a monolayer is compressed between two barriers

until it is a narrow collapsed film with a mean thickness of about 300 Å, it can be removed from the surface and the dry film can be examined by electron diffraction[1]. Considerable orientation develops during compression, and the diffraction pattern shows the principal features of an orientated α-helical structure, including a meridional reflexion at $1{\cdot}495 \pm 0{\cdot}005$ Å and a strong equatorial reflexion at 12·6 Å. Orientated air-dried specimens, for examination by polarized infrared spectroscopy, were each prepared by folding up a similar narrow collapsed film of polymer on the water surface and lifting it off on a silver chloride plate. The infrared dichroism and frequencies of the principal amide absorption bands were found to be those which are normally obtained for right-handed α-helices composed of L-residues. Furthermore, with very slight differences which are attributable to the differing side chain composition, they are almost identical to the spectrum obtained by Bradbury *et al.*[5] for the copolymer (86 per cent ethyl-L-aspartate : 14 per cent benzyl-L-aspartate) which they clearly show is from a right-handed α-helix. The spectrum produced by the left-handed α-helix is quite different in detail and it is reasonable to conclude from this evidence that specimens prepared from collapsed monolayers of poly-β-benzyl-L-aspartate are in the right-handed α-helical conformation. This conclusion is not surprising because many of the side chains, which by their interaction with the backbone normally cause the left-handed sense to be marginally more stable[6,7], will have less effect in the polar environment of the interface. It would therefore appear that, depending on the solvent used, a transition occurs to the right-handed α-helix from the left-handed α-helix or from the random-coil form, as the case may be, on spreading the monolayer.

Investigation by polarized infrared spectroscopy of orientated specimens prepared as already described has shown that the right-handed form has the following properties. (1) As with the left-handed form, heating an orientated specimen to 160° C causes a transition to the orientated ω-conformation. (2) While exposure to chloroform vapour has no effect, exposure of an orientated specimen for about 10 min to the vapour of chloroform containing 4 per cent by volume trifluoroacetic acid, or 10 per cent dichloroacetic acid, causes the polymer to undergo a transition to the orientated left-handed α-helical form. Prolonged exposure to the vapour (particularly when trifluoroacetic acid is used) or use of a concentration of acid which is too high causes the polymer to become disorientated. The film is swollen and softened by the acid and it would therefore be wrong to consider this conformational change to be strictly a solid state transition.

In view of the molecular weights of the polymers,

these conformational changes imply quite extensive molecular rearrangements. It might, however, be pointed out that to change the sense of the helix does not require a net rotation of the whole molecule about its axis; the transition can take place simply by breaking hydrogen bonds, together with rotations about the bonds to the αC atoms. The fact that the orientation of the molecules is not lost during the transition suggests that short regions first become unfolded, and that refolding in the opposite sense then takes place with a concomitant propagation of the unfolded regions along the molecules. The changes are therefore no more or less remarkable than the transition reported in orientated films of salts of poly-L-lysine where the α-helical structure can be converted to an orientated β-conformation by changes in the humidity[8,9].

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Surface Chemistry of Poly(β-benzyl L-Aspartate)

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Synopsis

Molecular monolayers of poly(β-benzyl L-aspartate) spread at an air–water interface have been studied. The results obtained both by direct observations on the monlayer and from examination of collapsed films with polarized infrared spectroscopy and electron diffraction are consistent with the presence of right handed α-helices in the monolayer when the molecular weight is high. When 1% (v/v) isopropanol is present in the subphase the right-handed helix prevails, provided that the monolayer is first spread on water. Monolayers of low molecular weight polymer appear to form the crossed-β structure. Orientated collapsed films of high molecular weight polymer can be converted to the left-handed α-helical and to the ω-conformation, and the mechanisms are discussed. The surface chemistry of this polymer is compared with that of related polymers and a consistent pattern of behavior emerges.

INTRODUCTION

Poly(β-benzyl L-aspartate) has been extensively investigated since it was discovered that in contrast with most synthetic polypeptides composed of L-residues it normally forms a left-handed rather than a right-handed α-helix.[1,2] Furthermore, heating a specimen to 160°C causes it to change to the 4.0_{13} ω-helix, which is also left-handed.[3] By comparison with the right-handed α-helix, the left-handed form is relatively unstable. A number of factors are implicated. Studies of copolymers with other aspartate esters have suggested that entropy considerations,[4] electrostatic interactions between the side chain and backbone,[5] and solvent effects[6,7] may all be important. Direct studies of this polymer in water would be of considerable value, in view of the biological interest in the left-handed helix, but are precluded since it is not soluble in water. In this situation studies at the air–water interface of molecular monolayers provide a useful alternative approach.

No single experimental method at present available is adequate to determine with certainty the detailed conformation of a polymer in the monolayer state. It is therefore necessary to proceed by combining direct studies on the monolayer with more powerful indirect techniques such as electron diffraction and polarized infrared spectroscopy which can be applied to collapsed films or multilayers removed from the surface. This approach has provided good evidence in support of the view that the α-helix is stable in a number of high molecular weight polypeptides when spread

as monolayers at the air–water interface.[8,9] The helices condense to form regularly packed arrays or micelles[9] which may contain many molecules.[10] Under pressure the monolayer progressively collapses to form a bilayer, giving rise to a plateau in the surface pressure–area curve, provided that the side chain is sufficiently long. The height of the plateau depends on the hydrophobic character of the side chain and on the free energy of the polymer–vapor interface. This model provides a basis for understanding peptide deuterium exchange[9] in the monolayer state and the series of transitions, indicative of the consecutive formation of several layers of molecules, which occur when a monolayer of poly-ϵ-benzyloxycarbonyl-L-lysine is compressed.[10] These results are not readily interpretable if the polymer is in an extended conformation in the monolayer state or if the plateau is associated with a reorientation of the side chains or some other conformational change.

From this background, the results from poly(β-benzyl L-aspartate) can be examined. It has already been reported that films prepared from collapsed monolayers are in the right-handed α-helical conformation,[11] and this result will here be considered further in relation to the properties of the monolayer.

EXPERIMENTAL

Material

Polymer specimens were obtained from the Sigma Chemical Company and Pilot Chemicals Incorporated (Lot A30) with molecular weights given as 130,000 and 250,000, respectively. A specimen of low molecular weight (5000 from the van Slyke method) was obtained from Yeda Research and Development Co. Ltd.

The specific viscosities of the two high molecular weight polymers measured at 0.2% (w/v) in dichloroacetic acid (25°C) were 0.17 (Sigma) and 0.32 (Pilot).

Dichloracetic acid (B.D.H.) was redistilled under reduced pressure, and Analar grade chloroform was distilled shortly prior to use. Water for the Langmuir trough was distilled twice, the second time from an all-glass still containing alkaline potassium permanganate. It had a conductivity of 700,000 ohm/cm or higher.

Methods

Solutions for spreading monolayers were normally made by dissolving about 10 mg of polymer in 1 ml dichloroacetic acid and making up to 10 ml volume with chloroform. About 0.03 ml was applied to a well-swept water surface in the Langmuir trough. About ten small drops were just touched on the surface from the tip of an all-glass Agla micrometer syringe at an area of 25 Å^2/residue. The trough was normally of fused silica 15 cm wide, and the monolayer was compressed at 4 mm/min by a continuous drive. The film balance was a simple flexure device.[12]

Oriented collapsed films were prepared for infrared spectroscopy and electron diffraction analysis as previously,[9,10] except for the low molecular weight specimen which produced very weak films. This necessitated a special method for preparing oriented films for spectroscopy, and for this a trough, 3 m long × 25 mm wide, was used. This was made of a piece of aluminum alloy channel which was cleaned and well waxed. The monolayer was spread in the usual manner and swept off by a barrier with a plate of silver chloride mounted on its leading edge. The collapse was effected in stages of about 30 cm of trough length, by lifting off a second barrier and moving it back along the trough as the film piled up against the silver chloride plate. At the end of the trough the film could be picked off on the plate and dried.

A two-part trough made of Perspex was used to investigate the effect of isopropanol in the subphase. This had an overall size of 60 × 15 cm, and was well waxed. A series of four narrow barriers across the width in the middle divided the trough into two main compartments and allowed limited mixing between them. These came to within 1 mm of the liquid surface. A complete seal, to keep separate different subphases, could be made by two waxed glass barriers with a 1-mm projection on the lower surface which fitted on top of the lower barriers. When these were removed, the liquid surface was continuous and a monolayer could be moved from one part to the other between two barriers. Stirring was effected by a magnetic roller which moved along the floor of the trough.

RESULTS

Surface Pressure-Area Curves

Both high molecular weight specimens behaved in the same way in all respects. The first steep rise of the surface pressure–area curve (Fig. 1) gives an area of 20.5 Å²/residue when extrapolated to zero pressure. The length of the plateau and the presence of the small initial peak depended to some extent on the spreading conditions. The plateau was shorter and the peak smaller if the monolayer was spread at an area greatly in excess of the condensed area or if the solution was applied to the surface in a large number of drops. The normal procedure was to apply about 10 drops at an area of 25 Å²/residue. The height of the plateau, which does not depend on the spreading conditions is 10.5 ± 0.5 dyne/cm (20°C) and about 2.5 dyne/cm lower at 40°C.

There appears to be no significant difference between monolayers spread from solution in chloroform containing 1% (v/v) dichloracetic acid, in which the polymer is α-helical and from chloroform with 10% acid in which it is randomly coiled.[1]

The low molecular weight material behaved quite differently: the curve rose less steeply from a higher initial area and had a smaller pressure at low areas. This shows that the film develops less strength than the high molecular weight material when it collapses, as might be expected. This

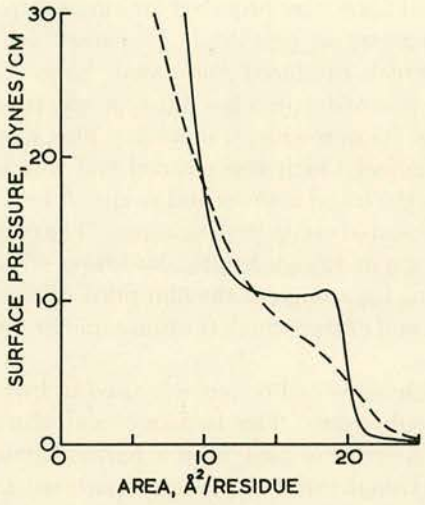

Fig. 1. Surface pressure–area curves for monolayers spread on water at 20°C: (——) high molecular weight; (---) low molecular weight.

was also evident from the difficulty experienced in removing collapsed material from the surface.

Infrared Spectra

An orientated specimen of the high molecular weight polymer prepared by collapsing a monolayer spread on distilled water gives a spectrum of a type not previously reported for this polymer (Fig. 2). It is however very similar to the spectrum of the copolymer (86% ethyl L-aspartate–14% benzyl L-aspartate) which Bradbury et al.[13] show is produced by a right-handed α-helical conformation. The frequencies of the ester band (1740 cm^{-1}), the amide I band (1658 cm^{-1}), and the amide II band (1552 cm^{-1}) were checked by measurements carried out at P.C.M.U. Harwell and are probably accurate to ± 2 cm^{-1}. These figures are in good agreement with those for the right-handed helix in the copolymer[13] and well outside the values given for the left-handed helix of poly(β-benzyl L-aspartate),[13] as also observed in this work on specimens prepared in the usual way.[3] The dichroic character of the ester C—O at 1168 cm^{-1} is a further check, since it is parallel for the right handed form but perpendicular for the left handed form. The relative intensities of the bands in the 1200–1300 cm^{-1} range are a further valuable diagnostic feature and are in good agreement with the spectrum of the right-handed copolymer.[13]

If a specimen prepared in a similar manner is suitably treated it can be converted to the left-handed α-helical or to the ω-conformation with practically no loss of orientation, as shown by the dichroism. The left-handed helix is produced by exposing the dry film to the vapor of chloroform containing 10% (v/v) dichloroacetic acid for about 10 min. (The chloroform vapor has no effect on its own, its use is to reduce the partial vapor pressure

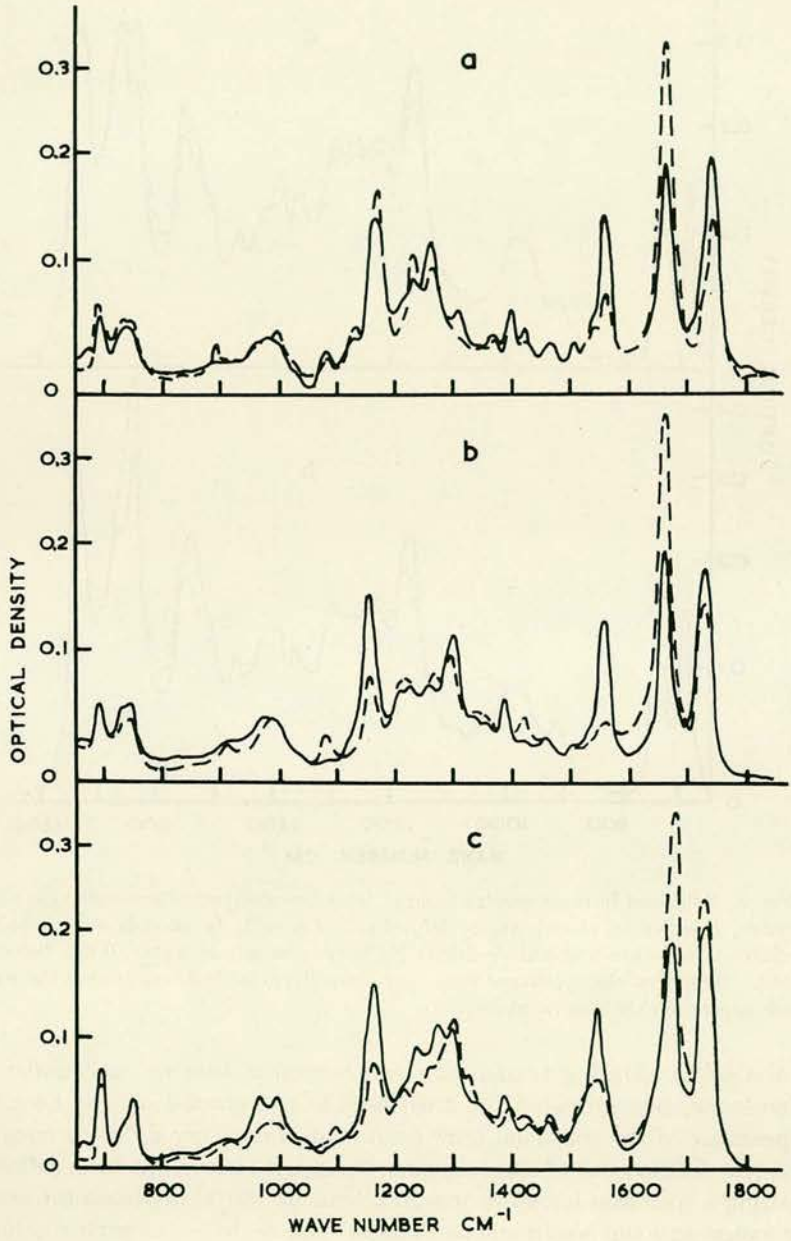

Fig. 2. Polarized infrared spectra from collapsed monolayers of high molecular weight polymer with (——) E vector perpendicular, (---) E vector parallel to specimen (i.e., parallel to barrier used to collapse the film): (*a*) air-dried; (*b*) air-dried, then exposed to vapor of chloroform containing 10% (v/v) dichloroacetic acid; (*c*) air-dried then heated briefly to 160°C.

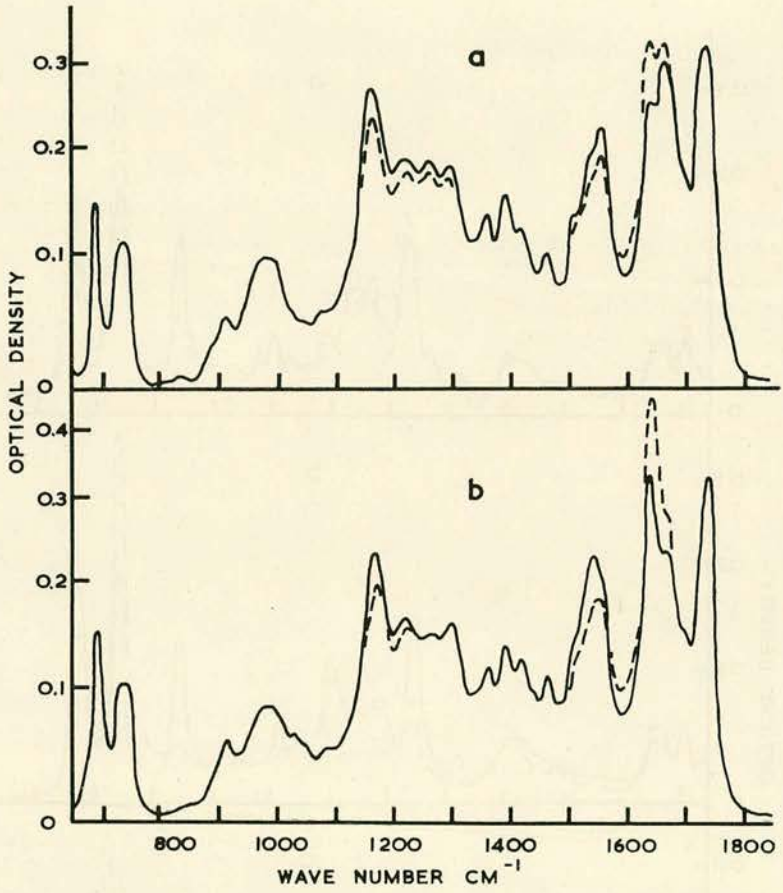

Fig. 3. Polarized infrared spectra from collapsed monolayers of low molecular weight polymer, directions of electric vector defined as in Figure 2: (*a*) monolayer removed immediately after spreading and air-dried; (*b*) monolayer left on water 70 min before removal. Note that the specimens were very nonuniform in thickness so that the strong bands appear weaker than normally.

of the acid.) During treatment the specimen is seen to swell under the microscope, but provided the treatment is not carried on too long, the appearance of the specimen after treatment is not very different once the acid has diffused out of the specimen, though it clearly has been softened. Heating a specimen for a few minutes in air at 160°C produces the ω-conformation and the birefringence changes from + to −, as with specimens prepared by heating the left-handed form. Again, it is clear that the material softens during treatment.

The spectrum of the low molecular weight material (Fig. 3) depends on the time the monolayer is left on the water surface. The low-frequency component (1635 cm^{-1}) of the amide I band increased with time over about the first hour after the monolayer is spread. This is a good indication that

a β-conformation was developing on the surface from α-helical or random coil material, which gives rise to the high-frequency component. This effect was first observed by Anderson,[14] who participated in the early stages of this work. In contrast to the high molecular weight material, the dichroism shows that the specimen forms with the molecular axes in line with the direction of compression rather than at right angles to it, and parallel to the barrier, as normally. This can be accounted for if it is assumed the molecules pack side-by-side, forming a β-conformation in which the longest dimension in the micelles is at right angles to the molecular axes, in the direction of the hydrogen bonds. The structure is therefore crossed-β, which has been recorded before in this polymer[3] and is not uncommon when the molecular weight is low,[15] but it has not previously been observed in the monolayer state.

It was not possible to correlate the development of the β-conformation as indicated by the infrared spectra with the shape of the surface pressure–area curves. The infrared spectra showed a significant amount of the β-conformation when the film was immediately removed and most of the increase appeared to take place in about the first 15 min. In this circumstance the surface pressure curve, which required about 30 min to record, was relatively insensitive.

Electron Diffraction Studies

Specimens for electron diffraction examination were prepared from collapsed films as described previously.[9] Air-dried specimens give diffraction patterns showing poor crystallinity but with a fair degree of fibre orientation. A strong equatorial reflection at about 12.5 Å, a very diffuse meridional arc at 1.52 Å (these differ somewhat from the earlier reported values[11]) and strong reflections in the region of 5.4 Å are the principal features of the pattern and are a good indication that the α-helix is present. If we take the 12.5 Å reflection to be the 100 reflection of a hexagonal cell, the density is 1.24 g/cc compared with 1.20 g/cc for the left-handed form.[3] In neither case, does the diffraction pattern enable a sense to be assigned to the helix.

If a specimen is heated briefly to 160°C there appears to be no loss of orientation in the specimen and the pattern changes to one with a high degree of crystallinity containing a number of well defined reflections. The pattern is very similar to that of the ω-conformation obtained by Bradbury et al.[3] with five reflections on the equator and four on the first layer line which agree in spacing with the strongest x-ray reflections. In addition there is a well defined reflection on the meridian at 5.30 Å, not recorded by Bradbury et al., which may be indexed as 001 on the unit cell they propose and which would be forbidden if all residues were equivalent. It was not, however, possible to find a structure in which all the side chains were similarly orientated and observation of this reflection is consistent with that conclusion. A similar situation exists with poly(γ-methyl L-glutamate) where electron diffraction reveals an 006 reflection.[16] These

results leave no doubt that the polymer conformation produced by heating collapsed monolayers is identical with the conformation produced by heating the left-handed α-helical structure.

Effect of Isopropanol in the Subphase

In the light of experience with other polymers it was anticipated that a number of factors might combine to affect the structure and behavior of the monolayer when isopropanol is added to the subphase. These are: (*1*) a lowering of the plateau arising principally from a reduction of the adhesion between the polymer and the subphase as is generally observed,[9] (*2*) a modification of the spreading arising from the lower surface tension of the subphase, (*3*) the conformation in the monolayer might be modified as a result of the lower polarity of the subphase, (*4*) the presence of isopropanol in the collapsed film might cause conformational changes after the film has been removed.

If a monolayer is spread directly on a subphase containing 1% (v/v) isopropanol there is no indication of a plateau (Fig. 4), and the collapsed film gives a spectrum characteristic of the left-handed α-helix. Exact reproducibility of the area is difficult, an indication that spreading conditions are critical, but it is invariably too low for any reasonable conformation in a monomolecular layer. Reduction of the isopropanol concentration to $^1/_2$% (v/v) produces a higher area at a given pressure and the spectrum suggests a mixture of the right- and left-handed forms. In contrast, if a monolayer is first spread on water and then very slowly transferred to a subphase containing 1% (v/v) isopropanol, a plateau is observed at about

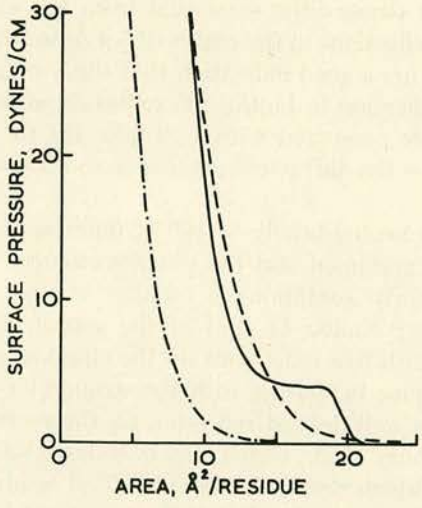

Fig. 4. Surface pressure–area curves for high molecular weight polymer on isopropanol–water mixtures: (–·–) on 1% (v/v) isopropanol; (—) on $^1/_2$% (v/v) isopropanol; (——) polymer first spread on distilled water and then transferred to 1% (v/v) isopropanol.

3 dyne/cm, the area is higher, and the spectrum is that of the right-handed α-helix. This is a good indication that isopropanol present in the film after removal of the film from the surface does not produce the left-handed helix and that, provided the right-handed helix is first formed in a monolayer, it is stable in the presence of 1% isopropanol in the subphase. The low plateau shows that the monolayer is only marginally stable and this is probably why spreading directly on isopropanol solutions gave poor reproducibility and low areas, for an excess surface pressure of 3 dyne is sufficient to force a molecule out of the surface. It is quite likely that during spreading such pressures are exceeded, since this is a nonequilibrium phenomenon with chloroform sweeping across the surface tending to compress any polymer already present. The final structure might well be very irregular with parts of individual molecules in contact with the subphase and other parts in a disorganized upper layer. In this situation it is unlikely that a molecule would be stable if it were to adopt left- and right-handed helical structures along its length, depending on the local environment, and the left-handed α-helix would then be favored. This explanation is consistent with the gradual initial rise in the pressure-area curve when the monolayer is spread directly on isopropanol solutions, compared with the case where the film is first spread on water, which shows the molecules are not fully condensed into an organized structure. In addition, when these monolayers are examined by electron diffraction and polarized infrared spectroscopy, little orientation and very low crystallinity is observed, as is to be expected from the collapse of a disordered structure.

Measurement of Angle of Contract

The angle of contact θ of the high molecular weight polymer was measured by the dipping-plate technique. A film was prepared by drying a solution in chloroform on a microscope slide. As in earlier work[9] the angle varied a few degrees across the specimen and was lower after the surface had been wet. The best value after wetting was 71 ± 2°. This may be compared with 73° for poly(γ-benzyl L-glutamate).[9] Since the polymer was in contact with water it is not possible to say whether the surface molecules were in the left-handed α-helical conformation, as prepared, or in the right-handed helix, but the two values of θ are unlikely to be very different.

DISCUSSION

Conformation of the Monolayer Spread on Water

The experimental results show quite clearly that the film of high molecular weight polymer when removed from the surface and dried down, is in the right-handed α-helical conformation, irrespective of whether the polymer in the spreading solution is in the left-handed helical form or is randomly coiled. A conformational change must therefore take place either during the spreading or removal of the film from the water surface

(or at both stages). A conformational change to the right-handed helix is very unlikely when the polymer is being removed and dried down, since the left-handed helix appears to be the more stable in the solid state, and our ability to convert the polymer to the left-handed form by exposure to dichloroacetic acid vapor supports this view. The most simple hypothesis is therefore that the right-handed helix forms during spreading of the monolayer. A more elaborate hypothesis is that some other conformation in the monolayer forms the right-handed helix on collapse at the plateau, but there is no evidence for this: on the contrary, the pattern of behavior in the monolayer follows that of earlier work.[9,17,18] This showed that the plateau can be accounted for very simply by the progressive collapse into a bilayer of molecules packed side-by-side in an orderly manner, as is to be expected from the rod-like nature of the helix. A further check is provided by the condensed monolayer area 20.5 Å^2/residue, which is in reasonable agreement with the area of 22 Å^2/residue calculated assuming the same interhelix distance as in the solid state (from the diffraction data). In addition, in view of the observation that when low molecular weight polymer is spread as a monolayer the infrared spectrum shows a progressive increase in the amount of β-structure, it is very probable that a β-structure would be similarly detected in the high molecular weight material if it were present, or formed during collapse or removal of the film. Therefore in the absence of contrary evidence or any method which provides detailed conformational information directly on the monolayer *in situ*, these results support the view that high molecular weight polymer forms an α-helix when spread as a monolayer and that it is right-handed.

The presence of the right-handed helix in the monolayer can be accounted for by the polar effect of the aqueous substrate weakening some of the side-chain backbone interactions important in causing the left-handed structure to be normally the more stable. In this context it is of interest to compare this polymer with poly(γ-benzyl L-glutamate), which has one —CH_2— more in the side chain. This causes the angle of contact of the cast film to be about 2° higher and the plateau about 4 dyne/cm lower. Similarly poly(β-methyl L-aspartate) has an inflection at about 25 dyne/cm[19] and poly(γ-methyl L-glutamate) a plateau at 20 dyne/cm[9]; again, poly-δ-benzyloxycarbonyl-L-ornithine[19] has a plateau at 10 dyne/cm and poly-ϵ-benzyloxycarbonyl-L-lysine[10] its first plateau at 6 dyne/cm. From these correlations it follows that in all three cases addition of one —CH_2— group to the β-C is the direct cause of the lowering of the plateau by 4–5 dyne/cm, and this is consistent with the explanation already given for the significance of the height of the plateau. The lowering of the plateau might partly arise from the free energy of the polymer–vapor interface being increased by the additional —CH_2— but more probably, because of the increased angle of contact and because the group is close to the backbone, the principal effect of the group is to reduce the adhesion of the polymer to the subphase by direct hydrophobic interaction with the water. A simple calculation suggests that this explanation is reasonable. Assume that in a monolayer which forms a plateau at 20 Å^2/residue, $^1/_3$ of the residues are directed into

the subphase and the remaining $^2/_3$ form polymer–polymer contacts or are directed into the air. If addition of one —CH_2— group to those side chains which are directed into the water lowers the plateau by 4 dyne/cm, this corresponds to 346 cal/mole of residues directed into the water. This value may be compared with 600 to 800 cal/mole for the desorption of a —CH_2— group from the water surface in simple systems.[20] In view of the gross simplifying assumptions involved, these figures are sufficiently close to support the view that water molecules penetrate the side chains of possibly $^1/_3$ of the total number of residues in the monolayer as far as the β-C atom. This will greatly modify side-chain backbone interactions, and in the case of poly(β-benzyl L-aspartate) this is why the right-handed α-helix forms in the monolayer.

Conformational Changes in the Collapsed Film

The molecular rearrangement in the collapsed film from the right-handed to left-handed α-helix or to the ω-conformation without appreciable loss of orientation is quite striking. It has been pointed out that a change of sense does not necessarily involve a net rotation of the whole molecule about its axis[11] (which would require a very high activation energy) since if the hydrogen bonds along a short length of helix are opened, refolding of that length can taken place in the opposite sense simply by rotations about the bonds to the α-C atoms. Since the molecules do not become disorientated during the change, it is probable that the unfolded regions are propagated along the molecules from one or both ends, the energy for the transition being derived from the higher stability of the left handed form. The transition is complete in a few minutes and in practice it is probable that the rate limiting factor is the diffusion rate of acid through the thickness of the film (about 1μ).

The observation that with a similar degree of heat treatment orientated films of right- or left-handed α-helices can be converted to the ω-helix suggests that a common factor controls the transition in both cases, probably melting of the side chains. When there is no change of helix sense, an α to ω transition can take place either by the helix tightening to 4 residues per turn, which involves a relative rotation of the two ends of the molecule or by a similar process to that above where a group of hydrogen bonds open, involving no such rotation. But when there is a change of helix sense as well as pitch, any mechanism must involve opening of hydrogen bonds, and it follows that both the α to ω transitions may take place in the same way with the propagation of an unfolded region along the molecule. The process is, however, more complicated than a simple change of helix sense, as considered above, since the molecule also shortens by 0.2 Å per residue.

CONCLUSION

The hypothesis that the α-helix is stable in a number of polypeptides when spread as monolayers at the air–water interface was originally based on

surface areas, infrared frequencies of collapsed films and qualitative deuterium exchange data.[8] It has subsequently been supported by work on other polymers with a range of side chain lengths and has satisfactorily explained the presence and height of the plateau in the surface pressure–area curves[9] and the remarkable series of transitions observed in the case of poly-ε-benzyloxycarbonyl-L-lysine.[10] Alternative hypotheses concerning the conformation in the monolayer give rise to difficulties which require further postulates simply to explain one or more aspect of the experimental results. The surface chemistry of poly(β-benzyl L-aspartate) fits into the pattern established on related polymers and the evidence that the helix is right handed is strong and theoretically reasonable. However, unless some direct method is developed for giving detailed conformational information on monolayers *in situ*, an element of uncertainty remains. From the experimental results it is reasonable to suggest that, if it were possible to prepare this polymer as a component of a block copolymer soluble in water, that it would be in the right-handed conformation in aqueous solution. This is of particular biological interest in view of the attention that has been given to the occurrence of the left-handed helix in nonaqueous systems.

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Multilayer Formation by a Compressed Monolayer of Poly-ε-benzyloxycarbonyl-L-lysine

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Molecular monolayers of poly-ε-benzyloxycarbonyl-L-lysine were studied at the air/water interface. Deuterium-exchange measurements and the surface area of the monolayer are consistent with a structure consisting of condensed ordered arrays of α-helices. Collapsed films removed from the surface and air-dried were examined by polarized infrared spectroscopy and electron diffraction and found to consist of molecules in the α-helical conformation. There is no indication of a conformational change during compression of the monolayer, and a series of transitions found in the force–area curve are interpreted as the consecutive formation of additional layers of molecules. Some of the factors that influence this almost perfect plastic behaviour are discussed.

Recent investigations of the surface chemistry of high-molecular-weight synthetic polypeptides have provided good evidence to show that when prepared as monolayers at the air/water interface they frequently exist as condensed ordered arrays of α-helices (Malcolm, 1962, 1965, 1966, 1968*a*). In some instances the force–area curve has a plateau, which has been interpreted as the regular formation of a second layer of molecules (Malcolm, 1966, 1968*a*). The pressure at which this forms is a characteristic of the polymer and depends on the work of adhesion between the polymer and the substrate and on the free energy of the polymer/vapour interface. In a few cases it has been possible to infer the development of a third layer of molecules. It appears that a necessary condition for the formation of a plateau is that the side chains should be sufficiently long and contain groups that interact to promote a co-operative transition. Thus for polypeptides with hydrocarbon side chains only an inflexion is observed in the force–area curve unless the side chain has four or more carbon atoms. Plateaux are observed, however, with the methyl, ethyl and benzyl esters of poly-L-glutamic acid (Malcolm, 1968*a*) and with poly-β-benzyl-L-aspartate (Malcolm, 1968*b*). It therefore seemed probable that poly-ε-benzyloxycarbonyl-L-lysine would exhibit a similar pattern of behaviour and a plateau was indeed observed, but in this instance there is remarkable evidence for the regular consecutive formation of additional layers of molecules and it provides a noteworthy example of almost perfect molecular plasticity.

MATERIALS AND METHODS

The material used was obtained from Pilot Chemicals Inc., Watertown, Mass., U.S.A. (lot L–78; mol.wt. 250000). Solutions for spreading monolayers were prepared by dissolving about 10 mg. of polymer in 1 ml. of dichloroacetic acid and making up to 10 ml. volume with chloroform. In this solvent the polymer is in the α-helical conformation (Karasz, O'Reilly & Bair, 1965). For deuterium-exchange experiments the polymer was deuterated by using *O*-deuterated dichloroacetic acid, and benzene in place of chloroform. Monolayers were prepared with about 0·06 ml. of the solution. These procedures have been described previously (Malcolm, 1968*a*).

Force–area measurements. The type of film balance employed is particularly suitable for this work. It consists essentially of a thin bent metal strip mounted with its lower edge in the interface, forming a flexible barrier across the Langmuir trough (Malcolm & Davies, 1965). When the monolayer is compressed against it, the strip is displaced and exerts a steady reaction on the film. The displacement of the strip, which is measured with a microscope fitted with a micrometer movement, is a measure of the force on the film. The film was compressed continuously with a waxed glass barrier moving at 4 mm./min. and readings were taken at ½ min. intervals. Although the results described below could probably have been obtained with other forms of apparatus, the continuous pressure applied to the film by this method probably gives better results than the usual procedures, where the film is compressed in increments. The force–area curves obtained are essentially dynamic, but the main features of the results are not at all sensitive to the rate of compression.

Observations on collapsed films. It was found that, if a monolayer is compressed until it forms a narrow strip between two barriers, high orientation frequently develops,

the molecules becoming aligned parallel to the barriers. The film can then be removed and examined by electron diffraction (Malcolm, 1968*a*). This method was applied here and extended to provide thicker oriented specimens for examination by polarized infrared spectroscopy. For this a two-pronged fork, suitably supported at its upper end, is lowered into the water while the film is being compressed. When the barriers are about 3 mm. apart they are gently moved back, releasing the film, and the fork is rotated and moved across the water so that the polymer strip is wound around it, forming a series of loops on the water surface. The barriers can then be used, if necessary, to compress the loops, and the film is picked off the surface by drawing an AgCl plate through the interface between the prongs of the fork. Infrared spectra of dry films so obtained were recorded on a Unicam SP. 200 spectrometer fitted with a selenium polarizer and an NaCl prism.

Angle of contact. The angle of contact of the polymer against water was measured with the dipping-plate technique as described previously (Malcolm, 1968*a*). Films of polymer for this were cast on microscope slides from solution in chloroform containing about 40% (v/v) *NN*-dimethylformamide.

RESULTS

Force–area curve. Monolayers spread on 0·01 N-hydrochloric acid and on distilled water gave similar force–area curves within the limit of experimental error (Fig. 1). An alternative way of plotting the data is also shown, where the product FA is plotted against A (F in dynes cm.$^{-1}$, A in Å^2/residue). This is essentially an idealized stress–strain curve (Malcolm, 1966), and additionally has the practical advantage of enabling detail to be recognized in the steeply rising part of the force–area curve. Extrapolation of the first steep rise of the curves to zero gives an area of $24{\cdot}0 \pm 0{\cdot}5$ Å^2/residue for the monolayer.

Minima occur in the FA–A curve at areas of approx. 12·1, 8·4, 5·9, 4·3 and 3·4(?) Å^2/residue. These can be interpreted as corresponding to the completion of two, three, four, five and (possibly) six layers of molecules. When a layer is complete, FA is a direct measure of the stress being applied to the film (at other areas the film is of non-uniform thickness and the stress uneven). The peaks in the force–area curve are accentuated by lowering the temperature of the trough to 16° and almost disappear if measurements are made at 34°. Similarly, lowering of the rate of compression of the film produces smaller peaks. Such variations in the conditions do not, however, significantly alter the positions of the minima in the FA–A curve.

Infrared spectroscopy and deuterium exchange. The spectrum of a collapsed monolayer (Fig. 2*a*) agreed in detail with that obtained from an oriented specimen prepared by drying a drop of solution of the polymer in chloroform, the film being stroked at the same time with a glass rod to promote alignment of the molecules. The amide I band

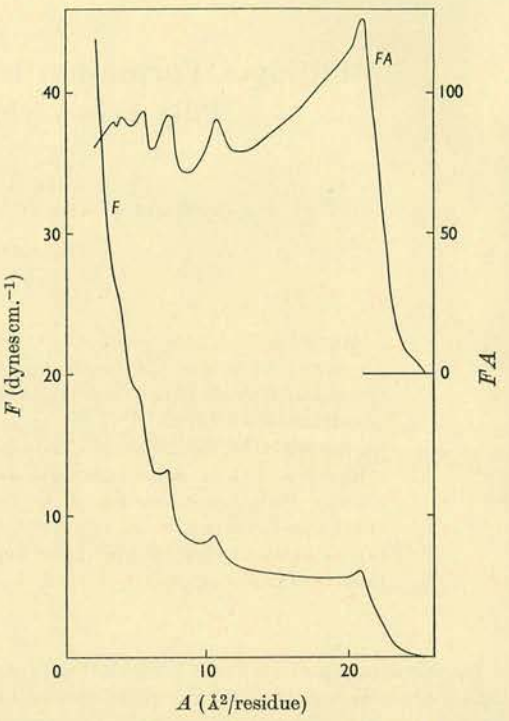

Fig. 1. Lower graph: dependence of area (A) on force (F) for a monolayer of poly-ε-benzyloxycarbonyl-L-lysine spread on 0·01 N-HCl (20°). Upper graph: plot of the product of the force (F) and the area (A) as a function of the area (A).

(1655 cm.$^{-1}$) has the frequency and dichroism normally found for α-helices; the corresponding band for the amide groups in the side chains is at 1700 cm.$^{-1}$ and has low dichroism. The NH stretching bands (approx. 3300 cm.$^{-1}$), the amide II band (approx. 1550 cm.$^{-1}$) and the amide III band (approx. 1250 cm.$^{-1}$) contain components from both the 'backbone' and side-chain amide groups. This is shown clearly from the spectrum of the deuterated specimen (Fig. 2*b*). The amide I band of the 'backbone' has moved to 1652 cm.$^{-1}$ and a dichroic band has appeared at 1450 cm.$^{-1}$, as is usually observed for deuterated α-helices (Miyazawa, 1962). On the other hand there remain bands at approx. 1700 cm.$^{-1}$, 3300 cm.$^{-1}$, 1540 cm.$^{-1}$ and 1250 cm.$^{-1}$, all of which now show little dichroism and are attributable to the side-chain amide group, assuming that the deuterium of the side-chain amide groups has completely exchanged. The absence of dichroism from these bands is probably a consequence of a measure of disorder and diversity of side-chain orientations, which is not unexpected and is probably correlated with the poor

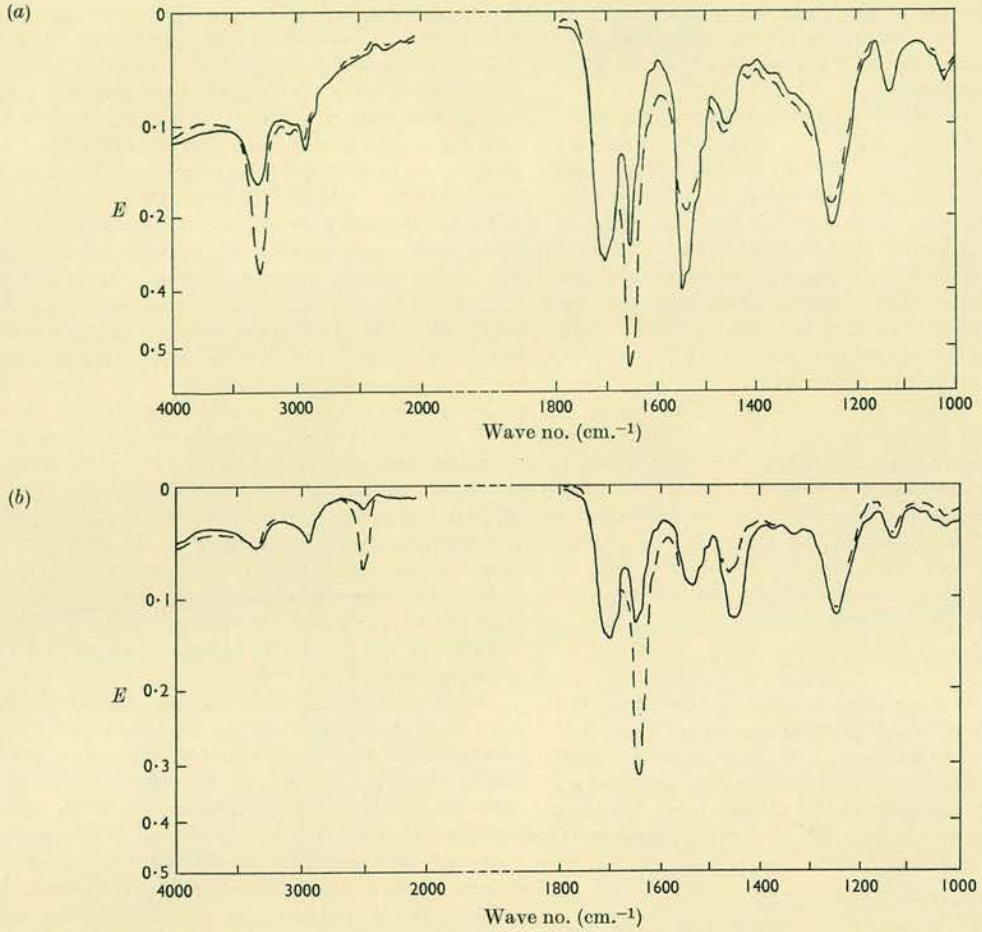

Fig. 2. Polarized infrared spectra of air-dried specimens prepared from monolayers (20°): (*a*) polymer spread on distilled water; (*b*) deuterated polymer spread on 0·01 N-HCl. ——, Electric vector perpendicular to the length of the specimen, i.e. parallel to the direction of compression of the film; — —, electric vector parallel to the specimen.

crystallinity of the electron-diffraction patterns (see below). The total time the polymer was in contact with water was about 15 min., and leaving a monolayer on the surface of 0·01 N-hydrochloric acid (20°) for 19 hr. produced little change in the spectrum. At a substrate pH 7·2 exchange is almost complete after 9 hr. and at pH 10 in a few minutes. Full kinetic measurements were not attempted, but the pattern clearly follows that observed in previous work, except that on alkaline substrates the exchange is rather more rapid than that observed in the esters of poly-L-glutamic acid (Malcolm, 1968*a*), probably on account of the hydrophilic amide group of the side chain promoting the exchange.

Electron diffraction. The diffraction patterns of air-dried specimens prepared from monolayers spread on distilled water show poor crystallinity but considerable orientation, with the usual intensity distribution characteristic of the α-helix, as observed in previous work (Malcolm, 1968*a*). There is a meridianal reflexion at 1·50 Å, and equatorial reflexions at approx. 14·3 Å and 8·2 Å that can be indexed as the (100) and (110) reflexions of a hexagonal cell. These two reflexions are of rather variable relative intensity from one specimen to another with the (100) usually weaker than the (110). This is somewhat similar to the situation found in poly-γ-methyl L-glutamate, where a high degree of double orientation occurs and the (100) reflexion, normally (in fibre photographs) by far the strongest, is not observed at all in some instances.

The explanation in this case is probably that, as with poly-γ-methyl L-glutamate, the structure develops with the (100) planes mainly parallel to the water surface.

Viewed in the electron microscope the films, estimated to be 200 Å in thickness, appeared remarkably uniform, except where folding had occurred in the last stages of collapse. This observation suggests that, although consecutive layers of molecules appear to form to a thickness of at least 50 Å, as shown by the force–area measurements, it is probable that this process continues further. Otherwise, if at 50 Å the film were to start to fold and buckle, the resulting texture would have been clearly visible in the microscope.

Angle of contact measurements. The angle of contact, θ, determined against distilled water, was $62 \pm 2°$. If we accept, bearing in mind the reservations that have been expressed (Malcolm, 1966, 1968*a*), the application of Young's equation as a measure of the adhesion of the polymer to the water, the following expression can be used to calculate the free energy of the polymer/vapour interface ($F_{P/V}$):

$$2F_{P/V} = -\gamma_{L/V}(1+\cos\theta) + W$$

where $\gamma_{L/V}$ is the surface tension of the water and W the work done on the film to convert 1 cm.$^{-2}$ of monolayer into a bilayer. W is numerically equal almost exactly (Malcolm, 1966) to the pressure on the film at the plateau (6 dynes cm.$^{-1}$). Taking 72·8 dynes cm.$^{-1}$ for $\gamma_{L/V}$, $2F_{P/V} = 101 \pm 2$ ergs cm.$^{-2}$.

DISCUSSION

The results of the polarized infrared spectroscopy and electron diffraction leave no doubt that when the fully collapsed polymer film is removed from the surface and air-dried it is in the α-helical conformation. If the force–area curve arises from the collapse of molecules in the α-helical conformation, the form of the curve can then be accounted for in a relatively simple way. Caution is, however, necessary, since it is in principle possible for some other conformation in the monolayer state to be converted into the α-helical form on compression, giving rise to a plateau in the force–area curve. It is therefore essential to distinguish between observations related directly to the structure of the monolayer and indirect observations on the collapsed film or a dried monolayer.

Loeb & Baier (1968) have used infrared multiple-reflexion spectra to study the conformation of poly-γ-benzyl L-glutamate monolayers. Although this approach is valuable, it is indirect since it involves transferring the monolayer to a germanium or other optical surface. This transfer may cause a conformational change, particularly if the monolayer interacts strongly with the water (as with poly-β-benzyl L-aspartate; Malcolm, 1968*b*) or is hydrogen-bonded to it. If it is possible to observe slow deuterium exchange, as in the present work, information can be obtained about the hydrogen-bonding in the monolayer. Early work (Malcolm, 1962) has, however, been questioned by Loeb & Baier (1967), who point out that deuterium–hydrogen exchange is complicated by the effect of the environment on the exchanging group and that the effect of the nearby surface has not been independently investigated. The kinetic measurements (Malcolm, 1968*a*) are, however, good support for this approach. In addition in the present work, the evidently complete exchange of the side-chain amide groups under conditions where the 'backbone' peptide groups do not exchange serves as a useful control, and shows that the two groups must be in different environments. If the polymer were a random coil on the surface, with all the groups free to hydrogen-bond to water, it would be expected that they would all rapidly exchange. It can therefore be concluded that, as in previous work, the slow exchange of the 'backbone' rules out conformations in which it hydrogen-bonds to the substrate water.

The interhelix distance in the collapsed film calculated assuming that the equatorial reflexions arise from the (100) and (110) planes of a hexagonal lattice is 16·4 Å. This is in reasonable agreement with the distance (16 Å) calculated from the area/residue measured in the monolayer, assuming the molecules are α-helical. It should be realized of course, particularly with long side chains, that the packing in a monolayer may not be the same as in the solid state, so that exact agreement is not necessarily to be expected. The observed area/residue is too high for the β-conformation to be acceptable, since this leads to an area in a monolayer of about 17 Å^2/residue (Malcolm, 1965).

The direct observations from both deuterium-exchange and surface-area measurement are therefore consistent with the presence of the α-helix in the monolayer, or of some closely related conformation. As in other cases (Malcolm, 1968*a*) the plateau implies a high degree of order in the monolayer, which on this basis arises directly from the side-by-side packing of the rigid helical molecules. On the other hand, if, as has been suggested for poly-γ-benzyl L-glutamate, a conformational change takes place at the plateau (Isemura & Suzuki, 1967) then in the present case some further explanation is necessary to account for the further inflexions in the force–area curves. The explanation given for the plateau in poly-γ-methyl L-glutamate (Malcolm, 1966) can be simply extended, however, by postulating the formation of further layers of molecules. If this is correct the monolayer micelles

of α-helices contain many molecules, since almost every micelle must form six or more layers on compression. A micelle might therefore contain about 100 or more molecules with an overall size of not less than 1500 Å × 1500 Å (assuming no end-to-end aggregation of the molecules). The formation of a multilayer, one layer at a time, can then take place by a simple co-operative process in the same way as suggested for the formation of a bilayer (Malcolm, 1968*a*), except that it is now necessary to postulate the movement upwards of molecules through the entire thickness of the film. Once, however, the pressure to form the first plateau is reached, where there is sufficient energy to detach molecules from the water surface, very little additional energy is necessary to propagate a movement of molecules through the film. An upper limit, bearing in mind that the measurements are dynamic (and the film therefore not in equilibrium), can be calculated from the force–area curve. For the third layer, the work done on the film above that required to remove molecules from the surface of the water is represented by the area in the force–area curve above the level of the first plateau, over the range where the third layer of molecules is forming. This gives approx. 1 erg for the formation of 1 cm.^{2} of triple layer or 0·03 kcal.mole^{-1} of residues transferred. Similarly, the peaks in the force–area curve may be related to the energy for initiation of the next layer of molecules (which requires more work than the propagation of a layer once it is initiated). In these regions, however, the film is clearly metastable and the size of the peaks is dependent on the experimental conditions as well as on the number and rate of formation of sites at which the next layer starts to form.

One factor important in determining the behaviour of the polymer is the free energy of the polymer/vapour interface, which is the main source of energy for the transition from monolayer to bilayer (Malcolm, 1968*a*). The value obtained is rather higher than in other cases where a plateau is observed, and it is of interest to compare this polymer with poly-γ-benzyl L-glutamate. This shows a plateau at about the same pressure and, as might be expected from the more hydrophobic nature of the side chain, has a significantly lower work of adhesion to water ($W_{P/L}$ 94 ergs cm.$^{-2}$). Thus although the amide group in the side chain of poly-ε-benzyloxycarbonyl-L-lysine increases the adhesion of the polymer to water, it correspondingly increases the free energy of the polymer/vapour interface. This relatively higher free energy might be one factor facilitating the formation of additional layers of molecules in the manner observed. There is, however, one important additional factor, since in this instance the side-chain amide groups at the polymer/vapour interface may adsorb water molecules by hydrogen-bonding. These may have to be displaced when a further layer of helices forms, so it cannot be assumed in this case that the free energy of the polymer/vapour interface is directly related to the energy required to separate two layers of polymer molecules. However, the presence of the water molecules could well be very important in promoting the almost perfect plasticity of the system.

In conclusion, it appears that this polymer fits into the pattern established in previous work, and in particular the remarkable formation of the multilayer is good support for the explanation that has been given earlier of the plateau. Although the side chain of this polymer is not one occurring in proteins it is nevertheless of interest as a model for understanding reactions, such as the deuterium-exchange reaction, at interfaces, and the factors that control the tertiary structure of proteins and intermolecular associations.

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PROTEIN CONFORMATIONS AND INTERACTIONS AT INTERFACES

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Monolayers of synthetic polypeptides and proteins have been investigated by infra-red spectroscopy and deuterium-exchange techniques. Poly-D-alanine is stable in the α-helical conformation at the air–water interface. The spectra of myoglobin and insulin show that unfolding of the protein does not lead to denaturation such as is produced by heating a solution and the evidence suggests that myoglobin probably loses only its tertiary structure at the air–water interface. The surface potential of poly-D-alanine is attributed to a net reorientation of the water molecules produced by spreading the monolayer.

Monolayers of α-helices are shown to pack with considerable side-chain interactions and the intermolecular forces could well be important in the structure and biochemistry of interfaces.

Introduction

OUR present concept of the molecular structure of a cell membrane owes much to Danielli & Harvey[1] who suggested that it consists of a bimolecular lipid layer with a layer of protein adsorbed on either side. This structure in various forms has received considerable support from electron microscopy and *X*-ray analysis;[2] it is also invoked to account for permeability properties in terms of a hypothetical molecular structure.[3] The protein layers are generally drawn as extended polypeptide chains with the side-chains interdigitating with the lipid. Sjostrand, for example, describes[4] the protein adjacent to the lipid as stretched polypeptide chains, but considers there to be an additional layer of globular protein on one side of the membrane in order to account for the asymmetry observed with the electron microscope.

The idea of extended polypeptide chains adjacent to the lipid receives support from experiments on the surface chemistry of proteins and synthetic polypeptides. In the case of poly-DL-leucine spread at the air–water interface, Cheesman & Davies[5] conclude that the side-chains are directed alternately into the air and down into the water; at the oil–water interface all the side-chains are supposed to be directed into the oil. The same authors state that the unfolding of myoglobin at a surface consists of a reorientation of

the polypeptide chain subsequent to the surmounting of an energy barrier due to a restriciting group. They consider the reorientation to be of a drastic nature involving a change of configuration throughout the chain, the bonds determining the configuration of the polypeptide chain being broken. They suggest that the interfacial unfolding of many, if not all, proteins involves a complete and irreversible change in the topochemistry of the polypeptide network, a prerequisite for this change being the removal of a restriction imposed either by a prosthetic group or by one or more intramolecular linkages between amino-acid residues. The situation at a lipid–water interface is not the same as at an oil–water interface because of the polar groups on the lipid molecules. Haydon & Taylor[6] have discussed this point considering again an extended polypeptide chain. They find that at a phospholipid–water interface, the energy required to uncoil a protein is too large for the free-energy change associated with the interpenetration of the extended chain or the side-chains into the phospholipid, and conclude that the protein would not therefore adsorb. At a cholesterol–water interface, unfolding and adsorption is considered a possibility.

It is difficult to reconcile the idea of a fully extended protein with the biochemical concept of a biological interface being the site of considerable enzyme activity. The most direct method of investigation is by *X*-ray analysis of membrane structures[7] and this approach has met with some success. Unfortunately, while the evidence supports the general model of the interface, the *X*-ray diagram lacks sufficient detail to provide a picture of the conformation of the proteins. This is not surprising, because of the diversity of the molecules present and because the protein is probably closely associated with the strongly scattering phosphate groups in the lipid. We are therefore led to reconsider the methods of surface chemistry and the conclusions that have been reached, in the light of progress in other fields. Bamford *et al.*[8] have discussed critically the surface chemistry of the synthetic polypeptides and have pointed out that in certain cases the results are inconclusive; they consider that often the α-helix of Pauling *et al.*[9] fits the experimental results as well, if not better, than an extended-chain structure. Evidence in support of this suggestion has been obtained from work on a number of synthetic polypeptides[10] and full details will be given elsewhere. If this is accepted, a reconsideration of the meaning of experiments using Langmuir trough, surface viscosity and surface potential techniques is necessary. It is important to settle these points beyond reasonable doubt on as wide a

range of polymers as possible. Further experiments are therefore described here on poly-alanine and by way of contrast on a nylon, and an attempt is made to extend the methods to monolayers of protein.

Poly-alanine has been chosen because it has been very fully studied in the solid state by *X*-rays[11,12] and infra-red spectroscopy[13] and in solution by optical rotation,[14] ultra-violet absorption[15] and deuterium exchange techniques.[16,17] A further important feature of this polymer is the small size of the side-chain, which helps to remove ambiguities in discussing alternative conformations. It will be shown that its behaviour in monolayers is again consistent with the presence of the α-helix. Part of the argument rests on infra-red spectroscopic measurements of deuterium–hydrogen exchange with the substrate, in monolayers in which the peptide hydrogen atoms have previously been replaced by deuterium. The exchange is slow under conditions which in solution would cause rapid exchange if the polymer were unfolded in a randomly coiled conformation. Because the chemical forces at an interface are not quite the same as in a solution, a direct comparison of exchange rates may not be valid. Analogous experiments have therefore been made on a nylon copolymer believed to be randomly coiled at the air–water interface.

The two proteins so far investigated, insulin and myoglobin, may not be typical of the proteins found in natural membranes. They are model systems from the point of view of this work and the conclusions reached on them have to be viewed in this light. However, our extensive knowledge of their structure at present outweighs this disadvantage.

Experimental

Force–area curves of monolayers were obtained in the usual manner with a Langmuir trough of fused silica, a waxed mica float and a torsion head sensitive to 0·04 dyne/cm./division. Surface potential measurements were made with a calomel/potassium chloride electrode in the trough and a 1-mc. polonium source above the surface coupled to a Lindemann electrometer and a potentiometer. Monolayers for spectroscopic examination were usually spread on a separate trough 60×15 cm.^{2} of waxed Perspex. To remove a monolayer, it was first compressed between two waxed glass barriers, until the separation between them was about 1·6 cm. The polymer was then removed by drawing a barium fluoride plate across the trough between the barriers. The crumpled multilayer so deposited on the plate could be lightly blotted and

dried down in a vacuum very rapidly to form a specimen in the form of a film 1 or 2 mm. wide across the plate. All solutions were spread from an all-glass Agla micrometer syringe as evenly as possible over the surface. The time taken to spread, remove, and dry the monolayer was about 2 min.

Infra-red spectra were obtained with a Unicam SP200 spectrometer immediately after the film had been dried. Care was taken that the specimen was accurately in the image plane of the spectrometer entrance-slit and its position was carefully adjusted for maximum absorption. The method of forming the specimen inevitably produces considerable non-uniformities in its thickness, nevertheless quantitative and fairly accurate measurements can be made of the deuterium exchange from the ND- and NH-bands. It is found that provided their optical density is not more than about 0·1, their relative strengths are not seriously affected. The percentage of amide hydrogen (%NH) is then calculated from the formula: $\%NH = NH/(NH+1{\cdot}33\ ND)\times 100\%$ where the symbols on the right represented the measured peak optical densities of the NH- and ND-bands at 3300 cm.$^{-1}$ and 2400 cm.$^{-1}$ respectively. The factor 1·33 arises because it has been found that the extinction coefficients of the NH- and ND-bands differ appreciably. An accurate value for this factor is difficult to obtain because of the problems involved in obtaining a uniform and fully deuterated specimen; the value given is probably correct to $\pm 0{\cdot}1$ for poly-D-alanine and the same value has been found for the 9 : 1 D–L-copolymer (see below). This uncertainty limits the absolute accuracy; relative measurements were reproducible usually to $\pm 2\%$. It should be noted that when the NH- and ND-bands are of about equal strength, errors caused by non-uniformity of the specimen and mis-alignment of the image of the specimen on the spectrometer slit are very small, since both bands are nearly equally affected. The formula assumes that the optical density of the NH- or ND-band in a specimen is proportional to the respective content of amide hydrogen or deuterium. If the vibrations in the molecule are coupled, as they appear to be,[18] this is not necessarily so. However experiments on poly-γ-ethyl-L-glutamate, to be reported elsewhere, suggest that this assumption is valid.

Materials

The poly-D-alanine and a 9 : 1 copolymer of D- and L-alanine were both prepared by Mr. W. E. Hanby of Courtaulds Ltd. These were found to have reduced viscosities respectively of 2·98 and

1·58 dl./g. measured at 25° at 0·2% w/v concentrations in dichloroacetic acid. The lower viscosity of the 9 : 1 copolymer is probably not an indication of a lower molecular weight but of a lower proportion of polymer in the α-helical conformation in this solvent.[14] *N*-Deuterated polymer solutions were prepared by dissolving about 15 mg. of the polymer in 1 ml. of $CHCl_2$·COOD followed by the addition of 9 ml. of chloroform. The sequence of this preparation is important and instructive. The deuterium exchange proceeds rapidly in a solvent able to open hydrogen bonds;[19] subsequent addition of a non-polar solvent helps to form and stabilise the α-helix. The $CHCl_2$·COOD was prepared by twice distilling dichloroacetic acid from an equal volume of 99·8% D_2O and the chloroform was redistilled to reduce the ethanol content. The polymer solutions did not keep well and it was necessary to use them within 2 days to obtain consistent results.

The nylon used was a 1 : 1 : 1 copolymer of nylon 6, nylon 66 and nylon 11 made available by Mr. R. W. J. Williams of British Nylon Spinners. A similar deuteration procedure was used as for poly-alanine except that, in order to keep the polymer in solution, 1 ml. C_2H_5·OD was added with the chloroform. This solution was used immediately after preparation.

Sperm-whale myoglobin was used as supplied by Seravac Laboratories (Pty) Ltd. without further purification. It was spread from solution in a 1 : 4 : 5 mixture by volume of dichloroacetic acid/water/isopropanol. Six-times-recrystallised ox-insulin was provided by Mr. J. Agar of Boots Pure Drug Co. Ltd. Solutions for spreading monolayers were made by dissolving about 10 mg. in 5 ml. of 0·01 N-hydrochloric acid followed by the addition of isopropanol to 10 ml. total volume.

Results

Poly-alanine

The force–area curves (Fig. 1) and surface potentials of poly-D-alanine and the 9 : 1 D-L-copolymer are so very similar that the differences are within the experimental errors and average values are given here. On both 0·01 N-hydrochloric acid and 0·01 N-sodium hydroxide the area per residue obtained by extrapolation of the almost linear part of the force–area curve to zero area is (13·8 ± 0·4)Å^2 per residue. This is good evidence that the structure of the monlayer is independent of pH. The surface potentials at this area are 550 mV on 0·01 N-hydrochloric acid and 515 mV on 0·01 N-sodium hydroxide, all measurements being taken at 20°. Before the film is compressed, the surface potential fluctuates widely at

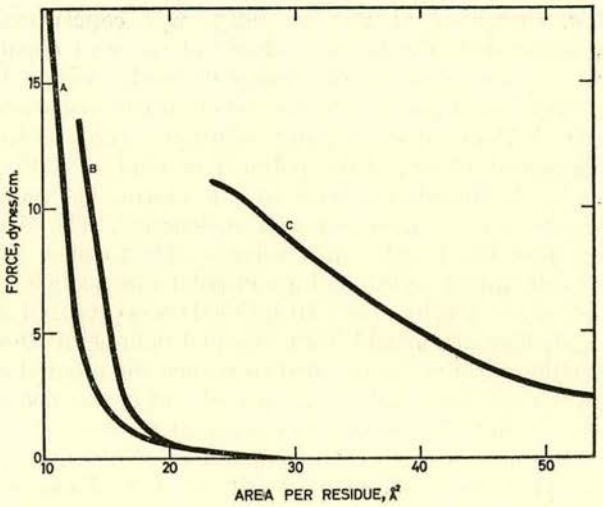

FIG. 1. *Force–area curves at the air–water interface* 20°
Poly-D-alanine (curve A) and nylon (curve C) on 0·01 N-HCl, and myoglobin (curve B) on 0·005 M-phosphate buffer pH 6·3

various parts of the surface showing that the film is condensed into large-scale aggregates. On compression of the monolayer the potential gradually becomes uniform over the surface. There is always a slight drift in the torsion-head reading each time the film is compressed. This almost certainly indicates a rearrangement of the molecules forming the aggregates and causes the precise shape of the force-area curve to be time-dependent. However by taking readings over about 30 min., this has only a slight effect on the force–area curves.

Fig. 2 shows the spectrum of poly-D-alanine (undeuterated) obtained by removing a monolayer which has been spread on dilute sodium hydroxide; very similar spectra are obtained spreading monolayers in the pH range 2–11. At higher pH values, the presence of sodium hydroxide on the film when it is dried causes difficulties. A typical spectrum obtained from a monolayer of *N*-deuterated 9 : 1, D–L-poly-alanine is shown in Fig. 2 (lower diagram). Because of the scatter of radiation caused by the non-uniformity of the specimen, the true zero for measurements of the ND- and NH-bands at 2400 cm.$^{-1}$ and 3300 cm.$^{-1}$ is estimated by drawing a straight line across from 2200 cm.$^{-1}$ to 3700 cm.$^{-1}$.

By measuring the deuterium content in the way described, for monolayers which have been on the surface for various lengths of

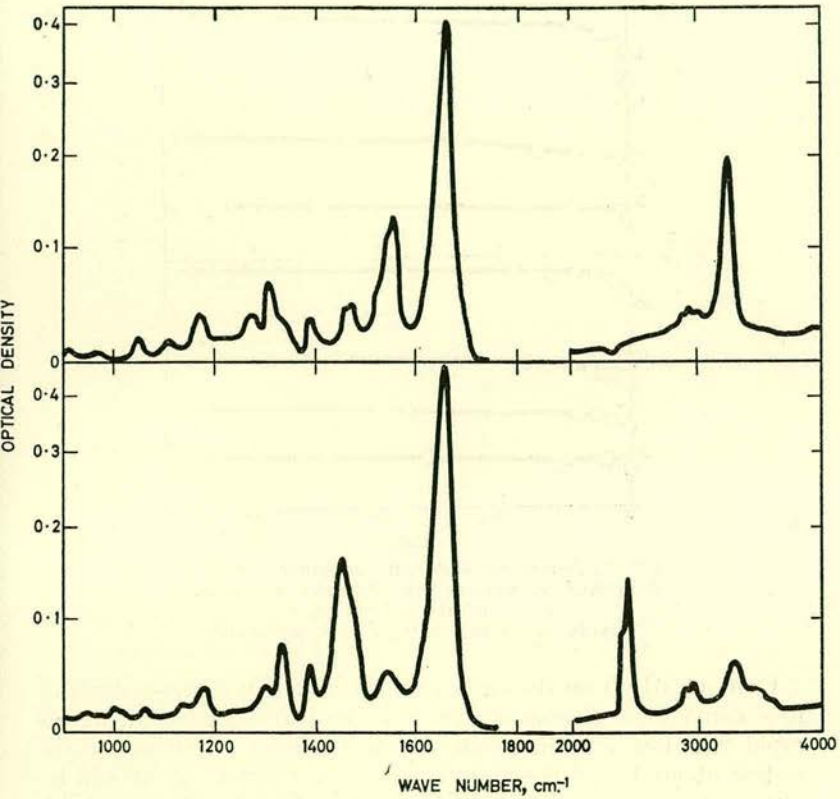

FIG. 2. (Upper diagram) *Infra-red spectrum of poly-*D*-alanine after being spread as a monolayer on* NaOH *pH* 10·5, 10 *min.*, 20°. (Lower diagram) N-*deuterated* 9 : 1 D–L *poly-alanine after being spread as a monolayer on HCl pH* 3, 10 *min.*, 22°

time, it is possible to investigate the rate of exchange under various conditions and make a comparison between the two polymers. As might be expected, the rate of exchange increases appreciably if the temperature is raised and in this work is has been kept constant at 22°. In the range 20 to 30Å² per residue, there is no observable dependence of the exchange rate on area (in contrast with poly-γ-methyl-L-glutamate) and all films were spread at 20 Å² per residue to make full use of the trough area. The exchange was timed from the moment of completion of spreading the film (Fig. 3). For both polymers the minimum rate of exhange was around pH 3. The results show that the initial deuterium content of the polymers must have been at least 85% and possible higher.

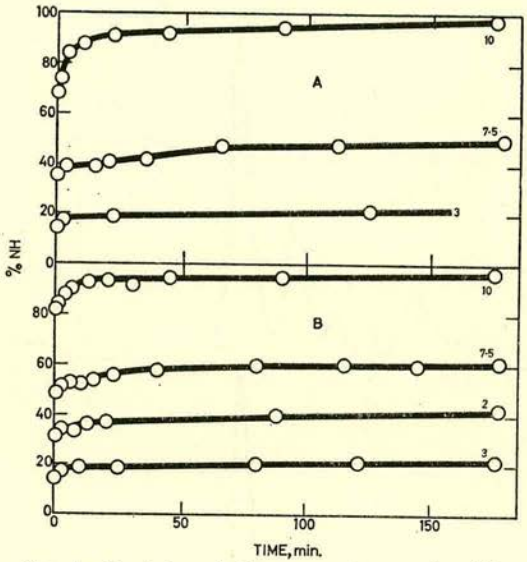

FIG. 3. *Deuterium–hydrogen exchange in* N-*deuterated monolayers on substrates at various constant values of pH* (22°)
A poly-D-alanine B 9 : 1 D–L-poly-alanine

Quite clearly from the curves the reaction is not first-order and it is convenient to consider there to be three phases, an initial rapid reaction over in about the first minute, a slower phase lasting about 1 h., followed by a very slow reaction which can be observed over many hours. The main difference between the exchange of poly-D-alanine and the 9 : 1 copolymer is in the extent of the initial exchange. This was investigated separately by preparing both solutions from the same solvents so that as far as possible conditions were the same. The exchange taking place in the first minute plotted against pH of the substrate is shown in Fig. 4. This shows that the incorporation of some L-residues in a molecule composed of D-residues increases the initial exchange rate, except, it appears, at pH 2–3.

Nylon monolayers were examined when spread on 0·01 N-hydrochloric acid. The force–area curve has been plotted taking a mean residue weight (190). When the *N*-deuterated polymer was spread on 0·01 N-acid at 22° and immediately removed, the spectrum was normal and showed no trace of any band attributable to deuterium. Because the spreading solution used was dilute and contained dichloroacetic acid which is very difficult to remove, it

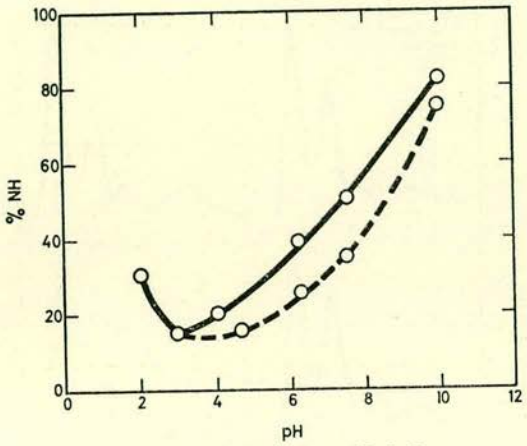

FIG. 4. *Measurements of the amide hydrogen content of* N-*deuterated polymer monolayers after* 1 *min. at different values of pH*
—— 9 : 1 D-L-poly-alanine
- - - poly-D-alanine

was not possible to show directly that the polymer was deuterated before spreading, but there is no reason to suppose that the chemistry of the exchange reaction in the preparation is any different from that of the polypeptides.

Monolayers of protein were found to be more difficult to remove than those of the synthetic polypeptides and it was necessary to have the substrate fairly close to neutrality. In order to compare the spectra of monolayers with those of the native protein and of denatured protein, spectra have been prepared (Fig. 5). The spectrum of the native protein was obtained by drying down at room temperature an aqueous solution of the protein spread evenly on a barium fluoride plate. The spectrum of denatured myoglobin was obtained by drying down in the same way the rather more viscous solution obtained by heating the protein solution to 90°. This treatment is not sufficient to denature insulin; but by heating to 100° a solution at pH 2 (or slightly lower), a gel starts to form after a few minutes and this can be spread into a film and dried down on barium fluoride. The spectra obtained by denaturing in this way show marked changes in the amide band at 1660 $cm.^{-1}$, with the development of a component of about equal strength at 1635 $cm.^{-1}$ in the case of myoglobin, and in insulin an almost complete shift of the band to 1635 $cm.^{-1}$. So far, spectra showing similar features have not been obtained by spreading monolayers

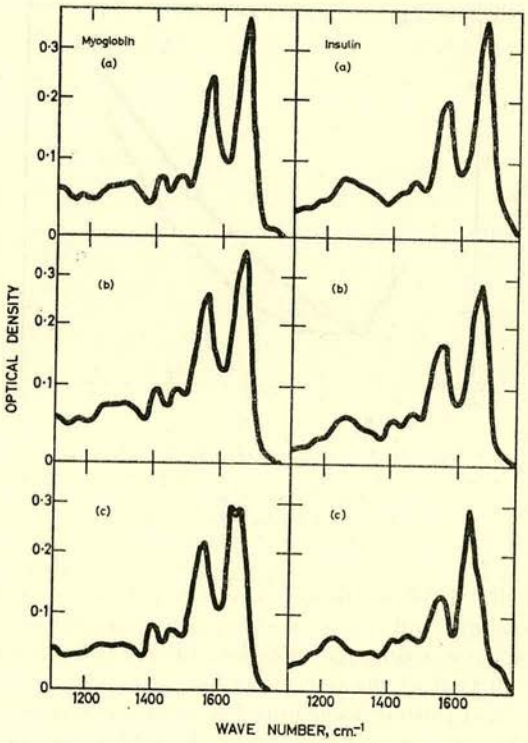

FIG. 5. *Infra-red spectra of myoglobin and insulin*
Myoglobin: (*a*) native protein, (*b*) monolayer after 45 min. on phosphate buffer pH 6·3, 20°, (*c*) cast from aqueous solution after 15 min. at 90°
Insulin: (*a*) native protein, (*b*) monolayer after 20 min. at 36° on distilled water, (*c*) after heating a solution in HCl pH 1·8, 15 min. at 100°

of protein. The actual appearance of the specimens of insulin monolayers spread on distilled water at 36° is different from those spread at 20° so that changes may well occur on heating monolayers, but the spectra are clearly more similar to the native than the denatured protein.

Discussion

In interpreting the experimental results we can consider three types of possible conformations in monolayers which are distinguished by their patterns of hydrogen-bonding:

(i) monolayers in which the peptide groups are hydrogen-bonded intramolecularly, as for example in the α-helix;

(ii) extended chain conformations with intermolecular hydrogen-bonds as in β-keratin;

(iii) extended chains with hydrogen-bonds formed between the amide groups and water molecules in the environment; these structures will be much more flexible because they lack the constraints of intra- or inter-molecular bonding. The term 'hydrogen-bond' will be used irrespective of whether the atom involved happens to be hydrogen or deuterium.

It will be assumed that the amide group is planar as postulated by Corey & Pauling,[20] so that the only free rotation in the polypeptide chain is around the bonds to the α-carbon atom. This assumption is supported by very considerable *X*-ray evidence and it has an important bearing on possible conformations in monolayers. If an attempt is made to build, with suitable models using this restriction, a structure in which all the bonds between the αC- and βC-atoms are directed to one side of a plane, it will be found impossible unless there is a fairly regular sequence of L- and D-residues; otherwise the molecule becomes very convoluted and no longer lies in the plane. Thus, the conformations proposed by Davies[21] for polypeptides with hydrocarbon side-chains in which all the hydrocarbon groups are directed to one side of an interface are only acceptable for copolymers of D- and L-residues and cannot be generalised to include polymers consisting of only one enantiomorph.

Considering now the spectra of poly-alanine (Fig. 2), these leave little doubt that the specimens are in the α-helical conformation after being removed from the interface and dried down. The spectrum of poly-D-alanine agrees in detail with the spectrum of α-poly-L-alanine shown by Elliott.[13] In particular there is no trace of the β-conformation of this polymer which is indicated by the shift of the amide band at 1660 cm.$^{-1}$ to 1634 cm.$^{-1}$ and other changes. It seems unlikely that if a film were in the β-conformation on the water surface it would spontaneously revert entirely to the α-form on being removed, since the β-conformation of this polymer appears to be quite stable; however, the spectrum by itself does not entirely rule this out nor does it eliminate the possibility that the molecule on the surface is unfolded and hydrogen-bonded to water. This latter possibility is the reason for the deuterium-exchange experiments.

We have good evidence that in solution poly-DL-alanine is

mainly randomly coiled[14,15,17] and deuterium-exchange rates indicate a first-order reaction.[16,17] The rate is slowest at pH 2·8 and at 20° the exchange is then complete within an hour. No observations have been reported with a pH above 4·91 since the exchange is then too rapid to follow by the technique of Berger & Linderstrom-Lang, even at 0°. The rates are of the same order as in simple di- and tri-peptides.[17] While these observations alone suggest that, if poly-D-alanine monolayers spread at an air–water interface were hydrogen-bonded to the water, exchange would be rather faster than is observed, the experiments with the nylon copolymer give additional support to this view. The particular polymer used was chosen because in it there are amide groups very similar to the amide group in poly-alanine, but separated by 4, 5, 6 or 10 methylene groups in nearly random order. In these circumstances intermolecular hydrogen-bonding is reduced to a minimum and it is reasonable to suppose, as in the discussion by Clark *et al.*,[22] that the amide groups bond to the water. The absence of any ND-band in the spectrum of the deuterated nylon polymer, after only 1 minute on a water surface at pH 2, is in marked contrast to the behaviour of poly-D-alanine and the 9 : 1 copolymer. The environment of the amide groups must be quite different in the two cases.

Turning now to the force–area curve of poly-D-alanine, its shape is typical of polypeptides with hydrocarbon side-chains. It has been found[12] that, in the solid state, α-helices in poly-L-alanine pack in a very neat and compact manner and it is a reasonable assumption (see below) that α-helices in a monolayer will pack in a similar fashion, particularly as the side-chains are small and inflexible. It can also be assumed that in a monolayer there will be a few regions of lower density where the helices do not pack too well because of their inflexibility. This will tend to increase the observed area per residue by a small amount. From the *X*-ray data,[11] the distance between α-helices is 8·55Å and the increment per residue along the helix 1·495Å giving an area per residue of 12·8Å^2. In the β-conformation the corresponding figures are 4·78Å for the inter-chain distance and 3·45Å for the residue increment measured along the chain, giving an area of 16·5Å^2 per residue. A repeat distance of 3·45Å is rather lower than the distance in a fully extended polypeptide chain and models show that the αC–βC bonds must be directed almost perpendicular to the plane of the molecules. It is therefore very compact and it is difficult to devise any other conformation, with more or less extended chains, which has a significantly lower area. The observed area per residue

of 13·8Å^2 is clearly consistent with the presence of α-helices but is too low for extended-chain structures.

The deuterium-exchange experiments are difficult to understand if it is supposed that the peptide groups are hydrogen-bonded to water. The problem is to account for the comparatively slow exchange after the very rapid exchange immediately the monolayer is spread. One possibility is to suppose that, after the monolayer has been on the surface for a short time, the structure is stabilised by intermolecular hydrogen-bonds, which might exchange more slowly, but as has been shown the observed area per residue is too small for this to be acceptable. The postulate that the α-helix is present provides the basis of an explanation that is reasonable and simple. When the monolayer is spread and the solvent has evaporated, the helices must rapidly form large crystalline aggregates. This is because a monolayer of rod-like molecules can only be packed in a restricted area if they form approximately parallel groups. Intermolecular forces are then sufficient to cause them to close-pack. The final structure is probably very similar to the packing of logs on a timber-river.

We can now broadly interpret the initial rapid phase of the exchange as occurring when the solvent has evaporated and the helices are at first isolated. Within this first minute, exchange will proceed at the ends of the helices, where the amide groups are more accessible or unbonded, and also as a result of thermal motion causing breaks within the helices. The second phase, when the monolayer has crystallised, is probably partly associated with exchange around the edges of the crystalline aggregates and partly exchange on the underside of the monolayer without the helices opening. The third very slow phase is then exchange at sites more removed from the water surface. Experiments still proceeding on monolayers under pressure, show that it is not possible to prevent exchange even when the molecules are closely packed. This supports the idea that it is not necessary for the helix to open for exchange to take place, a possibility considered by Berger & Linderstrom-Lang[16] in respect of solutions.

We know that the poly-alanine in solution is in the α-helical conformation;[14] there is then the possibility that it unfolds when first spread and then folds up again when the molecules form aggregates. The measurements of the initial exchange (Fig. 4) suggest this is unlikely. If the molecules were completely unfolded, L- and D-residues should exchange at the same rate and act independently. The effect of introducing a small proportion of L-residues on a helix composed of D-residues is either to make it less stable but

for its sense to be maintained, or for parts of the helix to have the opposite sense with consequent intervening breaks.[14] Either way, the effect is to introduce sites where exchange might be expected to occur more rapidly. It is then not surprising to find that there is a difference between the poly-D-alanine and the 9 : 1 D–L-copolymer which is mainly evident in the first phase of the reaction.

Monolayers of poly-DL-alanine have been investigated by Cumper & Alexander[23] who, from surface viscosity measurements, found an area per residue of 14·7Å^2 at both the air–water and oil–water interfaces and discuss the conformation in terms of an extended-chain structure. This work was however prior to the discovery of the α-helix. Later work by Glazer & Dogan[24] on the same polymer indicates a value of 14Å^2 per residue from the force–area curve and a surface potential of 540 mV at pH 2 falling to 410 mV at pH 12. They again consider the polymer to be unfolded, but the general agreement of all these values with those obtained in this work suggests that the α-helix is also stable in poly-DL-alanine. The variation of the surface potential with pH is attributed to changes in the state of ionisation of the end-groups and this may well be a contributory factor, but since appreciable changes with pH are found even with polypeptides of very high molecular weight, there may be some other cause. Davies[25] has related the variation of the surface potential with pH in polypeptides to the orientation of the C : O groups in the interface. This cannot be the correct explanation of the origin of the changes if the α-helix is present. With the exception of the ends of the molecule, it has no dipole-moment at right-angles to its axis, unless the side-chains orientate in some particular way with respect to the interface. This latter possibility is ruled out completely with poly-D-alanine and this present work therefore supports the earlier suggestion[10] that the potential arises from the water molecules. Alexander[26] has pointed out that we know nothing of the orientation of the water molecules at the air–water interface and that if we attempt to relate the surface potential to the orientation of the amide group in acetamides we find anomalies. A higher surface potential is found when the C : O groups of acetamides are parallel to the interface (as in the α-helix) than when they are perpendicular. If we attribute the surface potential to the water dipoles this anomaly can be resolved. One possibility is that the water molecules at the air–water interface have their hydrogen atoms more or less directed downwards and on spreading a monolayer the hydrogen atoms of the water tend to re-orientate towards the carbonyl oxygen atoms, perhaps forming a weak second hydrogen bond to the oxygen. A

slight dependence of this tendency on pH would account for the observed changes in monolayers in which the α-helix is present.

Considering now the monolayers of myoglobin and insulin, Ambrose & Elliott[27] have shown that denaturation of insulin by heating is accompanied by a shift in the amide-I band to 1637 cm.$^{-1}$ and other spectral changes, and that this correlated with the development of an extended-chain β-keratin type of structure. The changes in myoglobin suggest that about half the myoglobin is similarly converted on heating. The absence of corresponding bands in the spectra of monolayers is good evidence that any unfolding in the monolayers is not so extensive as to lead to the formation of a β-keratin structure. The observed area per residue in myoglobin 16·8Å^2, shows undoubtedly that some unfolding has taken place, since if the molecule maintained its globular form it would occupy not more than about 10Å^2 per residue. If we assume that the myoglobin monolayer is all folded as an α-helix and the inter-chain distance is about the same as in α-keratin, we can find the expected area per residue. The strong equatorial reflection at 9·8Å in α-keratin[28] can be considered, to a first approximation, as the $10\bar{1}0$ reflection of a hexagonal cell. This leads to an inter-helix distance of 11·3Å and an area per residue in a monolayer of 16·9Å^2. The close agreement of this value with the observed area in myoglobin is no doubt fortuitous, but, taken with the infra-red evidence, it supports the suggestion[10] that the unfolding observed with proteins at interfaces is often simply a loss of the tertiary structure. This would not be expected to affect significantly the infra-red spectrum. Since only just over two-thirds of the native myoglobin molecule is in the α-helical conformation,[29] the proportion in the monolayer may be no greater and there is not yet sufficient evidence to warrant further speculation.

Until more is known of the conformation of crystalline insulin, interpretation of the meaning of the area per residue, 15·5Å^2, is difficult. Unlike myoglobin it can have very little tertiary structure and the disulphide links probably maintain its form at the interface. Similarly until the origin of the surface potential in synthetic polypeptides is established, the values for myoglobin (340 mV) and insulin (180 mV) at the condensed areas would appear to have no precise significance.

The packing of α-helices in monolayers

Since the experimental results on poly-D-alanine strongly support the idea that the α-helix is stable at the air–water interface, and the data on myoglobin are consistent with this suggestion,

some of the consequences of the packing of α-helices will be discussed. The packing in a hexagonal lattice has been examined by Elliott & Malcolm[12] and their procedure and conclusions can be extended to monolayers. It is found that α-helices of poly-L-alanine pack in such a way that the positions of the β-carbon atoms of adjacent helices are equivalent, so that a translation of one helix in a direction at right-angles to its axis, along the cell axis direction, brings the β-carbon atoms into coincidence with those of an adjacent helix. The simplest case is for a helix with exactly 18 residues in 5 turns and this will be considered here, but the argument can be extended to totally irrational helices. When two helices are separated by the observed distance, this arrangement leads to the side-chains interpenetrating and forming a series of close contacts without appreciable strain. The situation in a monolayer is probably very similar and can be visualised as being derived by removing one layer of close-packed helices from the hexagonal lattice. This leads to 10 out of every 18 side-chains being in contact with adjacent helices. There are 4 in 18 side-chains directed into the air and the same number into the water. Since in a monolayer all the helices are already constrained to lie in a plane, and because the packing is equally good irrespective of the direction of the peptide sequence in adjacent molecules, this simple packing scheme must produce considerable cohesion and stability.

A possible way for α-helices of proteins to pack can be considered as an extension of this scheme. Considering first identical protein molecules, they will all tend to orientate in the same way with respect to the interface and their intermolecular association will be determined by the arrangement which gives rise to the maximum number of favourable side-chain interactions. This situation can be derived from the packing of poly-L-alanine by now considering the helices to be separated by the expected amount for proteins. This is shown in Fig. 6 in which 18 β-C atoms of three chains have been numbered successively and projected on a plane at rightangles to the axes of the chains. Consecutive numbers indicate a separation of 1·5 Å in a direction perpendicular to the plane of the drawing. The calculated distances between β-C atoms are as follows: 5 to 6 and 14 to 15, 6·6Å; 1 to 3, 1 to 17 (=1 to −1), 8 to 10 and 12 to 10, 6·05Å. These separations are such that, provided the chemical character of the side-chains is correct and the side-chains are of sufficient length, all the side-chains can interact. This of course implies a fairly specific sequence of the amino-acids on adjacent molecules. In a general way, however, this is reasonable for most protein monolayers since one side of the

molecule will usually have more hydrophilic groups than another which will orientate the molecule on the surface, so that there will always be a tendency for the hydrophobic groups to be associated or directed away from the water.

Similar interactions may play an important part in proteins at a lipid–water interface. The amino-acid sequence would then control the precise relationship of a protein molecule with its neighbours, while the remaining side-chains would interact with the lipid on one side of the layer and the aqueous phase on the other. This form of interaction is a feature of any protein conformation which has side-chains directed radially, as for example a globular protein. It is not a feature of the β-keratin type of structure since adjacent molecules are held together with hydrogen bonds; a translation of one molecule by about 6·9Å in the direction of its axis produces an equally favourable hydrogen-bonding situation. A fully extended protein molecule with peptide hydrogen-bonds formed with water is probably too flexible to lead to specific interactions and at a lipid surface might well be converted to an α-helical form; Doty[30] has shown that the helical content of proteins in solution is increased by decreasing the hydrogen-bonding capacity of the environment and the lipid surface might act in a similar manner.

There are several other general features of the α-helix as a membrane component. The position of the β-carbon atoms is fixed so that the side-chains are held in a fairly precise manner; in this way a hydrocarbon side-chain may occasionally be forced to remain in the aqueous phase where it might interact with hydrophobic groups in solution. The thickness of a monolayer of α-helices of protein is about 10 Å, which is reasonable, while the rigidity of the helix would confer considerable stability on the membrane. If a helix were to rotate about its axis as a result of chemical reactions it might act as a carrier for chemically specific translocations across the membrane.

While these features are attractive, it is important to realise that although there is a considerable proportion of the myoglobin and haemoglobin molecules in the α-helical fold, it may not be as extensive in the proteins composing the membrane. Finally, although myoglobin appears to lose its tertiary structure at the air–water interface, the behaviour of globular proteins at a lipid–water interface may be quite different; it cannot yet be ruled out that the tertiary structure may be partly maintained, though electron-microscope studies suggest a limited thickness for the protein layer.

Conclusions

The application of deuterium-exchange and infra-red techniques is clearly able to provide new information on the behaviour of polypeptide monolayers and shows that the α-helix is stable in monolayers of poly-D-alanine and 9 : 1, D–L-poly-alanine. This conclusion is consistent with the area per residue derived from experiments using the Langmuir trough comparing the values with those calculated from poly-L-alanine in the solid state. The difference between the initial deuterium exchange rates of poly-D-alanine and the 9 : 1 copolymer is evidence that the α-helices do not unfold completely and then re-form in the first stage of forming a monolayer (although partial unfolding cannot be ruled out). The general pattern of deuterium exchange is similar to that observed in several other polypeptides[10] and supports the view that the surface potential arises from a net reorientation of the water molecules when the monolayer is spread.

The spectra of insulin and myoglobin show no changes of the kind produced by thermal denaturation and the evidence suggests that the unfolding of myoglobin is mainly a loss of the tertiary structure, rather than a change of configuration throughout the chain as considered by Cheesman & Davies.[5]

The way in which α-helices can pack in poly-L-alanine and protein monolayers suggests that intermolecular forces can produce considerable stability; this may have important consequences in biology, particularly in relation to the structure and biochemistry of interfaces. There is clearly scope for many more experiments on both polypeptides and proteins, but it is satisfactory that a picture of the structure of monolayers can be obtained which is related to the structure in solution and in the crystalline state.

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Discussion

Dr. B. A. Pethica: Would not all the polypeptide be in contact with water, irrespective of its arrangement? This would appear to follow from the adsorption isotherms of water on proteins, etc., and the fact that the vapour is saturated at the surface.

Dr. B. R. Malcolm: Yes, this is probably true, but I do not think that it in any way alters my general arguments about the exchange reaction. If we take Fig. 6 to represent the packing of a monolayer

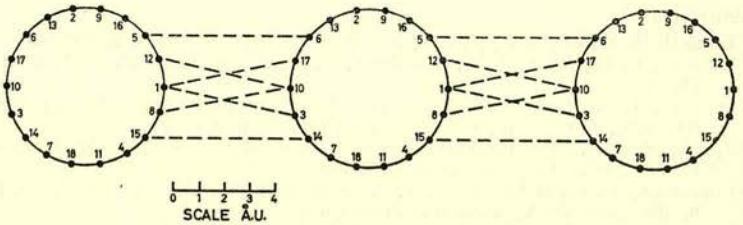

FIG. 6. *Packing of α-helices in proteins with β-carbon atoms in equivalent positions* Each β-carbon atom is numbered and projected on a plane perpendicular to the helix axis. Inter-helix distance 11·3 Å.

of poly-L-alanine, by reducing the inter-helix distance to 8·55Å, then the methyl groups with β-carbon atoms number 8, 1, 12, 3, 10 and 17 interpenetrate and are in close contact with those on adjacent helices. Because of the hydrophobic nature of these groups and for steric reasons, I would expect the corresponding amide deuterium to exchange more slowly than in the groups fully in the aqueous phase.

On the vapour side of the interface the exchange reaction is difficult to visualise. The first stage (as in the liquid) is probably the appearance of a charge somewhere on the amide group which will facilitate the exchange. I would expect this to be much less probable on the vapour than on the liquid side of the monolayer.

Sir Eric Rideal: There is evidence that enzymes become inactivated when brought to an interface and that solutions of albumen coagulate at rates proportional to their surface. Is that α–β conversion a result of adsorption or subsequent mechanical action?

Dr. B. R. Malcolm: The inactivation of an enzyme or the unfolding of a protein when at an interface does not necessarily, as I have tried to show, imply an α- to β-conversion in the sense I have defined the two forms. The monolayers of myoglobin undoubtedly coagulate which is why they can be removed readily from the water surface, but I think this arises from the action of the surface forces on the hydrophilic groups around the outside of the molecule. We know that in myoglobin a high proportion of the hydrophobic side-chains are buried inside the molecule; coagulation is probably caused by the intermolecular cohesion between these groups which must be exposed when the tertiary structure breaks down. How far this picture is true for albumen and other proteins remains to be seen.

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Protein Conformations in the Plasma Membrane

Abstract. *Infrared spectroscopy and optical rotatory dispersion have been used to test theories of structure of membrane protein. No evidence has been found to support the view that adjacent to the lipid there is a monolayer of protein in the β-conformation. The extracted protein appears to be a fairly typical globular protein with a low α-helical content.*

The classical Harvey-Danielli model (*1*) for the plasma membrane has formed the basis of discussion of its structure for the last 20 to 30 years. While the model was never intended to explain fully the varied behavior of the membrane, it remains the most acceptable basis for a theory of membrane structure. The state of the protein and the nature of its interaction with the lipid remain, however, almost totally unknown. The proposals of Danielli and his co-workers (*2*) were dependent on the current views on the conformation of proteins at interfaces. They cautiously concluded that proteins "on coming into contact with an oil-water interface, unroll into thin sheets, in a reversible manner" and that the membrane consisted of "a continuous film of lipoidal molecules, of which the two outermost layers are so orientated that the hydrated polar groups are in the oil-water interfaces, with a layer of protein molecules adsorbed on both of these interfaces" (*1*). Globular proteins were then believed to be adsorbed on these layers of "unrolled" or spreading proteins. Later workers have ascribed conformational states—usually the β-conformation—to the layers of protein associated with the lipid; for example, Kavanau (*3*) postulated that they "consist of unfolded and uncoiled fabric proteins in an extended β-conformation, possibly resembling a pleated sheet, with average spacings between the backbones of about 4.7 to 4.9 Å," together with some incompletely unfolded and uncoiled segments which provide regions of potential extensibility (*3a*).

The speculations on the arrangement of protein in the membrane have been greatly influenced by studies on the surface chemistry of proteins which implied that interfacial forces destroy the secondary and tertiary structure of proteins, producing interfacial films of unfolded open-chain molecules lacking any helical regions (*4*). However, infrared spectroscopy, and the measurement of rates of deuterium exchange in monolayers of synthetic polypeptides show that α-helices can exist at interfaces (*5*). Consequently, the whole question of the conformation of the protein in the membrane must be reopened bearing in mind that, since the conformation any protein adopts at any given time is contingent upon its environment, the conformation of membrane protein in vivo requires the presence of its associated lipid. As the techniques available for the study of conformation are mostly confined to substances in true solution or to crystals, the possibilities of a direct approach to membrane-protein conformation are limited.

Infrared spectroscopy is one of the few techniques yielding information on protein conformation that is applicable to the intact membrane, albeit in the form of a dry film. This technique reveals the presence of β-protein in the film by the position of the amide-I and -II bands which in the β state are observed at about 1630 and 1520 cm^{-1} as contrasted with about 1660 and 1540 cm^{-1} given by an α-helix or a random coil (*6*). The infrared spectrum of a film of hemoglobin-free ghosts of ox erythrocytes (*7*), dried in air at 20°C on a barium fluoride plate is shown in Fig. 1*a*. The amide-I band is at about 1660 cm^{-1} with no trace of a component at 1630 cm^{-1}. The amide-II band, while not so reliable for diagnostic purposes, also shows no indication of a β-conformation.

The ghost spectrum may be compared with that of a dry film prepared from a solution of the ghost protein obtained by *n*-butanol treatment of the ghost (*8*) (Fig. 1*b*, solid line). The differences between these two are attributable to the lipid component of the ghosts which can be estimated from a spectrum of the extracted lipid (Fig. 1*b*, broken line). The lipid makes only a small contribution to the spectrum of the ghost except in the region of the CH-stretching frequencies about 2850 cm^{-1}. About 50 percent of the extracted protein can be converted to a state possessing a β-form spectrum by heating with 50 percent ethanol at 70°C for 3 minutes (Fig. 1*c*).

It might be argued that the amount of β-protein required to form a layer on the lipid interface is, relative to other proteins in the membrane, too small to be detected by this method. That this is not the case is shown by calculation of the amount of β-protein required to cover the lipid of the membrane when the lipid is arranged as a bimolecular layer. The phospholipid, which makes up 70 percent of the total lipid, (28 percent of the ghost by weight) will—if it is assumed that each molecule in the bilayer occupies an area of 70 $Å^2$ (*9*) and has a mean molecular weight of 900—give a total surface area of $2.1 \times N$ $Å^2$ (N, Avogadro's number) per 100 g ghost material. The remaining 30 percent of the lipid is mostly cholesterol, which (if cross-sectional area is 36 $Å^2$ per molecule) can add a further maximum value of $1.1 \times N$ $Å^2$ to the total area of the lipid. The actual contribution of cholesterol is probably less in that (i) the area of a mixed monolayer of phospholipid and cholesterol is less than the sum of the areas of the two pure components (*10*) and (ii) the cholesterol molecules might undergo hydrogen-bonding together, giving a complex solvated in the hydrocarbon chains of the phospholipid bilayer. The second possibility seems to have been overlooked by many workers. We are therefore left with a total surface area for the lipid of about $3 \times N$ $Å^2$. As the area per amino acid residue of a protein monolayer is about 17 $Å^2$, in either α- or β-conformation, the protein, which accounts for about 60 percent by weight of the ghost, would (if the mean residue weight of the protein is 120) form a monolayer of $8.5 \times N$ $Å^2$ per 100 g ghost. Therefore, 35 percent of the protein of the ghost would have to be in the β-conformation to form a complete layer over the lipid; if present, this percentage is high enough to be detected by infrared spectroscopy. The protein layer contiguous with the lipid has been considered insoluble, yet over 90 percent of the total ghost protein may be obtained in aqueous solution by butanol treatment of the ghosts (*8*).

The infrared analysis, while it excludes a significant amount of the β-form, does not distinguish between the α-helix and the random chain; but the amount of α-helix in the membrane, or, to be more precise, the membrane protein when it is dissolved in water, can be estimated from the optical rotatory dispersion of the aqueous solution. Removal of lipid from the protein may well change the latter's conformation from the state in vivo, although neither the

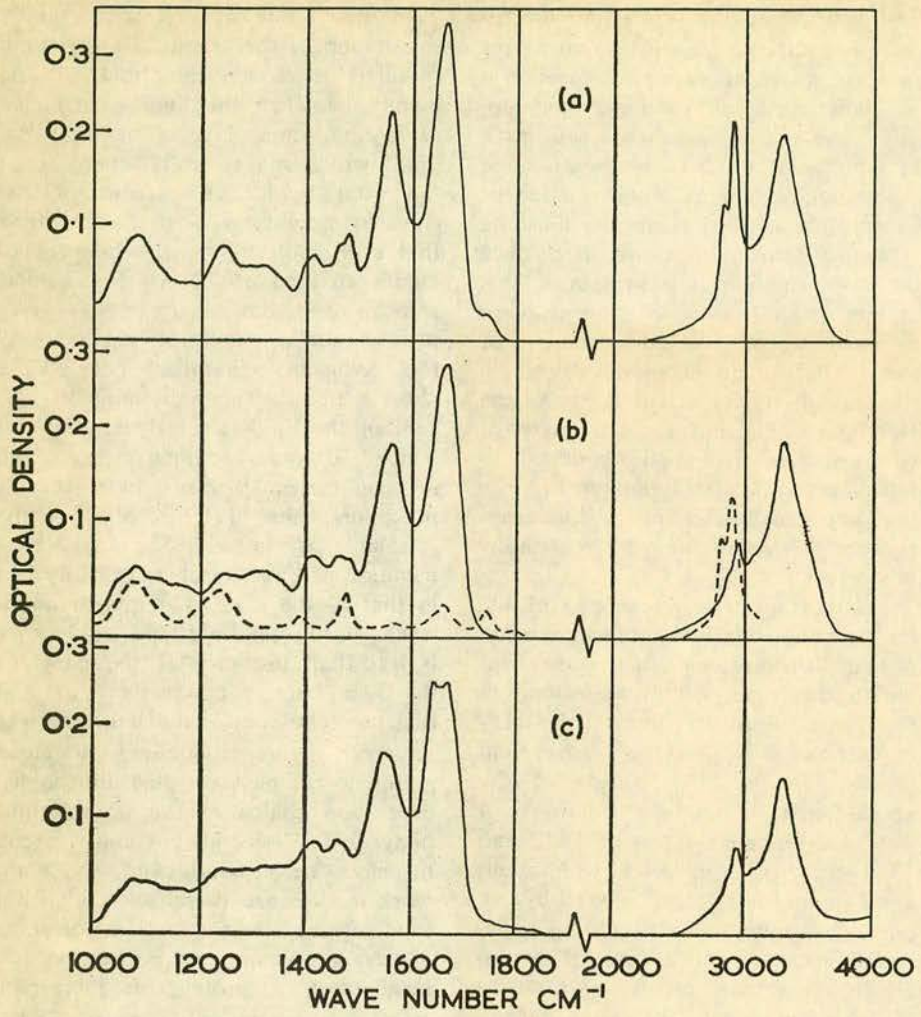

Fig. 1. Infrared spectra of air-dried specimens. (*a*) Red-cell ghosts. (*b*) Solid line, extracted ghost protein; broken line, estimated relative contribution of lipid to spectrum of ghost. (*c*) Ghost protein denatured with ethanol, showing about 50 percent of β-conformation.

butanol treatment nor the air drying used for preparing the infrared films would be expected to result in a decrease of any β-form which may have been present. Solutions of the protein in phosphate buffer at *p*H 7.0 (ionic strength 0.1) have therefore been examined with a polarimeter (Bellingham and Stanley Polarmatic 62) over the range 588 mμ to 246 mμ. The results can be fitted to the equation for proteins developed by Moffitt and Yang (*11*) with $\lambda_0 = 216$ mμ. For a mean residue weight of 120 the constant b_0, which can be interpreted as proportional to α-helical content, is found to be -90 compared to about -535 if the α-helix content was 100 percent, with $\lambda_0 = 216$ mμ. The most straightforward, but not unique, interpretation of these results is therefore that the protein contains about 17 percent of α-helix. However, the presence of about 8 percent by weight of sugars (sialic acid, hexosamine, and hexose), a trace of lipid, and possibly a small amount of the β-form not detectable by infrared spectroscopy, may cause this figure to be only a rough estimate.

These observations suggest that the membrane protein is • fairly typical globular protein perhaps modified by its sialic acid residues. How and to what extent the α-helical and random-coil components interact with the lipid still remain largely an open question, but the supposition of an extensive array of the protein in the β-conformation adjacent to the lipid is no longer justifiable. This removes a rather stable and perhaps intractable element from certain models, a trend in tune with recent concepts of the dynamic interrelation between different micellar states in the lipoidal phase.

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globulin synthesis should clarify the relationship of various classical types of lymphoid cells.

The synthesis of immunoglobulins by cultured lymphoblastoid cells derived from the buffy coats of patients with myeloid leukemias, lymphosarcomas, and Hodgkin's disease has been demonstrated in our laboratories (*2*). Over 50 cell lines originated from 26 patients are available for study. In contrast, no immunoglobulins have been detected in cell lines derived from malignancies of nonhemopoietic tissues.

We would appreciate the opportunity of obtaining blood samples from patients with multiple myeloma who have plasma cells in their peripheral blood.

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Protein Conformations in Biological Membranes

The conclusions of Maddy and Malcolm (*1*), based upon infrared spectroscopy of dried erythrocyte membranes and optical rotatory dispersion of solutions of membrane proteins, are unwarranted for the conformations in vivo of proteins in biological membranes. Their quotation from my book (*2*) and their added remarks do not convey the points that I propose an extended β-type conformation only for the primarily structural proteins of hydrated membranes in vivo (decidedly not for all the proteins, nor in dried membranes), and that this conformation is stabilized by interactions with both the lipid phase and the aqueous phase of the membrane (involving hydrophobic interactions as a major component). According to this treatment, one may expect a large decrease in the amount of extended conformation upon either dehydrating the membrane or greatly reducing its lipid content. In fact I discuss in detail (*2*) the total conversion of the extended conformation of the structural proteins to a globular conformation as a result of reduction or loss of the lipid phase under certain conditions, and the reversible partial conversion to the globular conformation during membrane transformations.

In the present state of our knowledge it is a step backward to conclude that findings regarding the conformations of proteins in dried membranes set guidelines for future studies and for theorizing concerning the conformations in vivo of membrane proteins. It would be interesting to know the basis for the authors' statement that air drying of a membrane should not be expected to reduce the amount of β-conformation present. For such an assertion to carry weight it should be based on detailed knowledge of either the structure of the membrane or the variation in β-conformation with drying in some pertinent lipid-protein model.

Maddy and Malcolm remark that many workers seem to have overlooked the possibility that cholesterol molecules may form hydrogen bonds with one another and dissolve in the hydrocarbon chains of the lipid phase; references are desirable to some of the treatments of membrane structure in which this possibility was not overlooked. The presence of proteins in extended conformations in biological membranes, far from being an "intractable element" relative to micellar transformations of the lipid phase, as Maddy and Malcolm assert, is an essential element of the only detailed theoretical treatment of such membrane transformations (*2*).

If the relevance of the highly indirect experimental approach of Maddy and Malcolm could be demonstrated first in some model system, say by elucidating the structure of the simple aqueous gelatin gel from structural studies of dried gelatin films, one might have a precedent for drawing conclusions regarding the structure of biological membranes in vivo from structural studies of dried membranes.

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3 January 1966

It should be clear from our paper that we do not exclude extended conformations, only the β-conformation, as an important structural feature of cell-membrane proteins. The term β-conformation refers to structures with extended or nearly extended polypeptide chains, each with a twofold screw axis and peptide hydrogen bonds between the chains (for example see *1*). The presence of this structure in silk and β-keratin, or in other proteins as a result of thermal or other denaturation processes, leads to a cross-linked structure insoluble in water; there has been no previous suggestion that it needed additional stabilizing forces. We showed that the proteins of the erythrocyte ghost are not exceptional in this respect, in that the β-form can be observed in dry denatured films. As to Kavanau's proposed stabilization by hydrophobic interactions, it is difficult to see how these occur between protein and lipid. Haydon and Taylor have pointed out the steric problems (*2*); if the protein were in the β-form these would be particularly severe, since the nonpolar side chains would not be long enough to penetrate beyond the polar lipid head-groups. This is a fundamental probem that Kavanau does not answer in his detailed theoretical treatment.

During further work we have obtained infrared spectra of fresh erythrocyte ghosts suspended in D_2O and of a solution of ghost protein in D_2O; both spectra show an amide I band which is symmetrical about 1648 cm^{-1} and which is unaffected by drying. Thermal denaturation, followed by drying of the protein, leads to a broader asymmetrical band with peaks at 1648 and 1632 cm^{-1}. We attribute the latter peak to formation of denatured β-protein: this peak is not detected in the unheated preparations.

These observations support our conclusion that there is no experimental foundation for the supposition of an extensive array of protein in the β-conformation adjacent to the lipid; they meet Kavanau's criticisms more directly than would experiments on gelatin. Since gelatin, with its high proline content, does not form the β-conformation it is difficult to see how it could settle the point at issue.

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STUDIES OF SYNTHETIC POLYPEPTIDE-WATER INTERACTIONS USING MONOLAYER TECHNIQUES

by

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INTRODUCTION

Early work on the surface chemistry of synthetic polypeptides spread as monolayers at the air-water interface was interpreted as indicating that the molecules were in an extended conformation, with the surface potential arising from the orientation of the peptide dipoles with respect to the interface[1]. There is however now considerable evidence that in many instances high molecular weight polymers form condensed ordered arrays of α-helices.[2-5] Since the helix backbone can have very little dipole moment at right angles to its axis, the surface potential attributable to it must arise almost entirely from interactions with the underlying water consequent upon spreading the monolayer,[2] An additional contribution to the potential can arise from side chain-water interactions. A range of polymers of related side chain composition has therefore been examined to see how the side chain influences the surface potential and surface pressure-area characteristics, with the expectation that this will increase our understanding of polymer-water interactions and the meaning of the surface potential.

Relatively thick orientated films of polymer can be prepared from collapsed monolayers.[4] This produces specimens in the α-helical conformation with very little polymer in the extended β-form. Provided that the polymer is not too hydrophobic, the adsorption of water from the vapour phase can be observed on such specimens by the use of polarized infrared spectroscopy.[6] The absorption attributable to the OH-stretching frequencies is dichroic, indicating a preferred orientation of the molecules, which probably arises from their interaction with the polymer at specific sites. Since the interpretation of the surface potential requires the postulate of similar interactions, it is of interest to see to what extent the two quite different types of observation might be related.

EXPERIMENTAL

Materials

Poly-L-Alanine was obtained from Sigma (Lot 107B-0599). Poly-γ-benzyl-D-glutamate (Poly Glu(OBzl), MW 235000, Lot G-108 and Poly-δ-benzyloxycarbonyl-L-ornithine (Poly Orn(Z)), MW 192000, Lot O-22, were obtained from Pilot Chemicals. The remaining polymers, poly-D-α-amino-n-butyric acid, poly-L-norvaline, poly-L-norleucine, poly-ε-benzyloxycarbonyl-L-lysine (MW 250000) (poly Lys(Z)), poly-β-benzyl-L-aspartate (MW 250000) (poly Asp(OBzl)), poly-γ-methyl-L-glutamate (polyGlu(OMe)) and poly-γ-ethyl-L-glutamate (polyGlu(OEt)) were as used previously.[2-5]

Measurements of the surface pressure and potential.

All monolayers were spread from solution in 10% dichloracetic acid-chloroform (v/v) at a concentration of approximately 1 mg/ml. The acid (B.D.H.) was redistilled under reduced pressure and Analar grade chloroform (B.D.H.) freshly distilled shortly prior to use. The subsolution, 0.01M KCl, was made up from twice distilled water and Analar grade KCl (B.D.H.) twice recrystallised. The Langmuir trough was of fused silica, 15cm wide. The film balance was a simple flexure device, based on the deflection of a bent metal strip in the interface which is followed with a microscope.[7] A helical potentiometer coupled to the micrometer drive of the microscope provides a signal for a recorder, directly proportional to the force on the film. Films for the surface potential measurements were normally compressed at a rate of 6.2 mm/min by a continuous drive and the potential was recorded automatically using a Curium-242 air ionizing electrode and a E.I.L.Vibron electrometer. Lower rates of compression, routinely 4 mm/min were used in separate experiments to record the surface pressure. At or below these rates, the curves were almost independent of the rate of compression. The higher rate of compression for the potential measurements minimised errors due to zero drift during the experiment. In other respects the procedures followed earlier work.[2-5]

Observation of the infrared spectra of water adsorbed on thick films

Three of the polmers, poly Ala, poly Glu(OMe) and poly Glu(OEt) are sufficiently hydrophylic for it to be possible to obtain spectra of water adsorbed on them from the vapour phase, in the region of the OH-stretching vibrations. Orientated specimens were prepared by collapsing monolayers.[4] While other methods may prove equally satisfactory this method has three advantages, (1) the molecules are orientated in the α-helical conformation by compression rather than by stretching which often causes part of the polymer to be in an extended chain conformation, (2) all traces of solvent are effectively removed, (3) N-deuterated specimens are readily prepared by spreading the deuterated polymer on 0.01 N HCl[2]: this provides a useful diminution in the strength of the Amide A (NH-stretching) band which lies close to the water band. Sufficient monolayers were collapsed to give specimens with an optical density of 1.5 or greater for the Amide I band. These were folded on the water surface and mounted on a barium fluoride plate. After drying the specimen in a vacuum a drop of a volatile liquid was applied to the surface to soften and compact the film and reduce the scatter of radiation (chloroform for poly Ala, 5% chloroform/benzene for the other polymers). Residual scatter was compensated for with MgO powder on a plate in the reference beam. The specimen was exposed to water vapour at various humidities from aqueous salt solutions in a cell, and also to methanol and ethanol vapour, all at 30°C. The absorption bands are weak and in any further study higher dispersion than that available with the NaCl prism used would be desirable.

RESULTS

Surface pressure-area measurements

The results are presented (Figure 1) in such a way that comparisons can be made between polymers of closely related chemical composition. Agreement is good with earlier measurements on 0.01 M HCl[2]. The initial steep rise of the curves is at an area consistent with a monolayer of close packed α-helices and the inflection or plateau arises from the collapse of the monolayer to form a bilayer.[2]

Poly Orn(Z) has not been previously investigated but in general it follows the same pattern of behaviour as poly Lys(Z).[4] However with one $-CH_2-$ less in the side chain, the curve rises at a lower initial area and shows only one further inflection after the plateau. Poly Lys(Z) differs in that inflections arise at areas consistent with the consecutive formation of 3,4 and 5 layers of molecules. Since the molecular weights of the two polymers are comparable, it appears that as with polymers with n-alkyl side chains, a longer side chain produces more clearly defined transitions in the pressure-area curve.

Poly Asp(OBzl) appears to form a right handed α-helix in the monolayer state, in contrast to the anomalous left handed helix it usually forms[5] and it can therefore be compared directly with poly Glu(OBzl). It will be seen that as with poly Orn(Z) and poly Lys(Z), an additional $-CH_2-$ in the side chain lowers the plateau.

Surface potential measurements

The general form of the curves follows that of earlier work[2] except at low areas, where the use of a continuous drive produces smother curves. Up to the plateau the potential V follows approximately the relation V x Area/residue = constant, for each polymer. Above the areas shown, irregularities are observed which indicate that the film is not uniformly distributed. Where the bilayer forms, the curves show plateaux with only slight curvature. Poly Lys(Z) has a further inflection, corresponding to the formation of additional layers of molecules, but the increase in height is small. The polymers with n-alkyl side chains shown plateaux in the potentials at heights depending on how close the molecules ultimately pack, but the range of potentials at 20 A^2/residue is relatively narrow (420-365mV).

The remaining four polymers all with ester groups in the side chains do not follow a regular pattern of behaviour. The surface potential of poly Asp(OBzl) is 200 mV higher than poly Glu(OBzl) and the potential of poly Glu(OEt) appears exceptionally high compared with poly Glu(OMe) and poly Glu(OBzl).

Infrared spectra of adsorbed water and alcohols

Spectra are shown for adsorption on poly Ala and poly Glu(OEt) (Figure 2). Poly Glu(OMe) appears to behave in the same way as poly Glu(OEt) and a spectrum has been given elsewhere.[6] During the course of an

experiment the relative strengths of the Amide A (NH-stretching) band (3300 cm^{-1}) and its N-deuterated counterpart (2450 cm^{-1}) changed slowly on account of exchange with the vapour (not shown for clarity). This appeared to have no significant effect on the OH-stretching bands in the 3400-3600 cm^{-1} region. Separate though similar specimens were used for the treatment with alcohol vapour. Attempts to observe absorption in the region of the OH deformation vibration were not successful on account of the strength of the Amide I band which overlies it, and there were no perceptible differences in the range down to 800 cm^{-1}. There was very little of the β-conformation detectable in any of the specimens as judged by the infrared spectra and in agreement with electron diffraction studies of specimens prepared in a similar manner[2]. Spectra were also obtained from monolayers containing 1:1 mixtures of poly Ala and Poly Glu(OMe) for comparison with hydrated keratin. Spectra from specimens which had not been N-deuterated had water bands essentially similar to those shown, though not so well resolved from the Amide A band.

It will be seen that while liquid water has a broad band centered about 3420 cm^{-1}, water absorbed on poly Ala has a band at approximately 3470cm^{-1} with a shoulder at 3530 cm^{-1} and poly Glu (OEt) water bands at 3440, 3510 and 3570 cm^{-1}. Methanol and ethanol both give one band in poly Ala at about 3400 cm^{-1}, not resolved from the residual Amide A band, and possibly for this reason showing very little dichroism, since the perpendicular dichroism is of opposite character to the Amide A band. The

The alcohols produce two bands showing marked perpendicular dichroism, at about 3450 and 3530 cm^{-1} in the glutamate polymers.

DISCUSSION

The surface pressure measurements

From the proposal that the plateau represents an orderly transition from a monolayer to a bilayer, its height is a measure of the work required for the transition and is determined by the work of adhesion between the polymer and substrate, and the free energy of the polymer-vapour interface γ_{pv} [2,3]. This can be seen subject to a number of simplifying assumptions as follows. When the bilayer forms under a pressure of W dyne/cm

$$\gamma_{lv} - \gamma_{pv} - \gamma_{pl} - W = 0 \qquad (1)$$

γ_{lv}, γ_{pv} and γ_{pl} refer to the free energy per unit area of the liquid-vapour, polymer-vapour and polymer-liquid interfaces. Combining (1) with Young's equation in the form

$$\gamma_{pl} = \gamma_{pv} - \gamma_{lv} \cos \Theta \qquad (2)$$

we get $$2\gamma_{pv} = \gamma_{lv} (1 + \cos \Theta) - W \qquad (3)$$

From this equation and assuming that we can use the same value for Θ as for a bulk specimen, γ_{pv} can be obtained. (1) assumes that the system is perfectly reversible, which has not been shown experimentally. However

a good indication that the system is not too far from perfect is shown by poly Lys(Z). In this instance it is possible to observe clearly the plateau corresponding to the formation of the third layer of molecules 3dyn/cm higher than the first plateau. On the basis of (1) it would be expected to remain at the same height and not therefore observable. That the additional work done to form the third layer is no more than 1.5 erg/cm^2 shows that the polymer behaves as an almost perfect plastic, and the application of (1) is not unreasonable as a first approximation in order to establish a general understanding of the monolayers. Furthermore the value obtained for γ_{pv} for poly Glu(OMe)[2] of 45.5 erg/cm^2, corresponds well with the range 40-50 dyn/cm for recent measurements of the critical surface tension of the polymer in the α-helical conformation.[8]

We can now understand the difference in the relative heights of the plateaux in poly Glu(OBzl) and poly Asp(OBzl) and in poly Lys(Z) and poly Orn(Z) where the virtual removal of one CH_2 from the side chain raises the plateau by 4 and 6 dyn/cm respectively. Both these pairs of polymers have bulky groups on the ends of the side chains which will determine the free energy of the polymer-vapour interface and which will therefore be almost the same for each one of the pair. The difference in side chain composition will then mainly affect the work of adhesion to water. If we assume that in a monolayer which is undergoing a transition to a bilayer at 20 Å^2/residue 1/3 or the side chains are surrounded by water,

4 and 6 dyn/cm correspond to energy differences of 345 and 517 cal/mole respectively for side chains being removed from the water. In view of the simplifying assumptions involved these figures are not unreasonable, compared with values of 600-800 cal/mole for the desorption of $-CH_2-$ groups from the air-water interface in simple systems.[9] This supports the general interpretation of the significance of the plateau and shows clearly an interaction between the water and a component of the side chain. Similar information might in principle be obtained from measurements of contact angles, but since the values obtained are only accurate to within 2° and show variations depending on how the specimens are prepared,[8] it is unlikely that significant differences would emerge between closely related polymers.

A similar correlation in the relative heights of the plateaux of poly Glu(OEt) and poly Glu(OMe) is not observed, but in this instance the difference in structure occurs close to the end of the side chain so that both γ_{pl} and γ_{pv} will be affected and corresponding behaviour is not therefore to be expected.

Interpretation of the Surface Potential

The surface potential-area curves all show a similar general shape with three main parts: (i) a steady rise varying inversely as the area, up to a point corresponding to the start of the plateau in the surface pressure curves, (ii) a region over which the potential is either constant or changing slowly, arising from the formation of the bilayer, (iii) a decrease

which becomes more steep as the monolayer approaches final collapse. It should be noted that the dependence of potential on area in stage (i) would not in general be expected if there were a conformational change during compression. Similarly, if the plateaux in the pressure-area curves were the result of a conformational change, as opposed to the formation of a bilayer, then the potential would in general be expected to increase or decrease during (ii).[2]

The various factors that contribute to the potential are related in principle to those that determine the static dielectric properties of helices in solution. We are however concerned with components of dipoles perpendicular to the interface with the added complexity compared with solutions, that the environment of the dipoles depends on their location with respect to the air-water interface. An empirical approach is necessary as a first step, treating the various factors that contribute to the potential arising from the backbone and the side chains as separable.

A long isolated helix has no net dipole moment perpendicular to its axis, since all non-axial components of the peptide dipole cancel out. It can therefore make no direct contribution to the potential. However, two factors, analogous to solvent effects in solution, can make contributions: (i) a dipolar contribution from the net reorientation of the water induced by spreading the monolayer, (ii) since peptide dipoles on the upper surface of the helix (in air) may have a different non-axial

dipole moment to those on the lower surface (in water) as a result of the differing dielectric constants of the environment, complete cancellation of the peptide dipoles may not occur. (ii) is probably small since the strongest component of the peptide dipole is axial, though the precise direction is difficult to determine since it is sensitive to assumptions made concerning polarization effects.[10] The experimental results show that (i) is predominant, since considering the series starting with poly Ala, there is only a slight dependence of the potential on side chain length (Fig. 1 curves a-d) at 20$\mathring{A}^2$/Residue, indicative of a specific peptide group-water dipolar interaction. If (ii) were significant, a marked dependence on side chain length would be expected, since the side chain would have the effect of altering the dielectric constant in the vicinity of the peptide group.

Considering how poly Orn(Z) and poly Lys(Z) at 22 $\mathring{A}^2$/Residue there is little difference in the surface potentials, suggesting that again the length of the hydrocarbon in the side chain has little effect on the potential. Since however the side chains now are flexible and contain polar groups, contributions to the potential can arise from both the regular helical array of side chains being distorted by the water and from a side chain-water interaction. If the backbone-water interaction is the same as that of the previous polymers, the net effect of the side chains is small and the same for both polymers.

The remaining four polymers, all with ester groups in the side chains, show that marked differences can be produced between related polymers by one $-CH_2-$ group in the side chain. The preceding discussions strongly suggest that this is not a direct consequence of the $-CH_2-$ group contributing to the potential. The differences therefore probably arise from polymer-water interactions which are sensitive to the side chain length and conformation. That these make a positive contribution to the potential is shown by the potentials being large compared with poly Ala (at the same area, say 20 Å^2/residue). A number of factors are involved. Studies of side chain conformations show that they are sensitive to the composition, the proximity of the ester group to the backbone and the dielectric constant of the environment.[11] Differing interactions between the side chains and water are therefore to be expected from one polymer to another, and in some cases a specific interaction such as an H_2O bridge between side chain ester and peptide group may be possible. Such dipolar and hydrogen bond interactions would be consistent with the preferred orientation of water molecules, as shown from the infrared dichroism. In addition the dielectric constant in the region of the side chains on the underside of the helices is different from that on the upper surface, hence even if their helical conformation is unmodified by the presence of the water, the side chains may contribute to a finite non-axial dipole moment perpendicular to the surface. That the dielectric constant is a significant factor fits in with the observation that while poly Asp(OBzl) is normally a left handed helix, it is right handed in the monolayer state on water and it has been

suggested that this arises from the water molecules weakening the side chain-backbone interactions which normally favour the left handed form.[5] Theoretical studies[11] suggest that the dielectric constant of the medium may be responsible for this, but specific interactions with water have not been ruled out.

Interpretation of the Infrared Spectra

While some of the details of the interpretation of the infrared spectra are necessarily tentative at this stage, a number of important general conclusions can be drawn with reasonable confidence. There are five aspects of the spectra which give information:

(1) It is possible to recognise two or three distinct components to the water absorption band, in contrast to the appearance of the band in liquid water or hydrated keratin, indicating a more ordered structure.

(2) The spectra are dichroic showing that the water molecules are on average orientated with respect to the polymer.

(3) The frequencies of the bands are higher than usual for a normal hydrogen bond with an OH group, but not high enough to assign them to completely unbonded OH groups. From the relation between hydrogen bond strength and frequency,[12] it appears therefore that the hydrogen bond component of the binding is weak.

(4) While the spectra of water adsorbed on poly Glu(OMe) and poly Glu(OEt) are similar, the spectrum of water adsorbed on poly Ala is

significantly different. This suggests the possibility of distinguishing between side chain and backbone interactions with the water.

(5) Broadly corresponding effects are observed using methanol and ethanol instead of water. This not only assists in the interpretation, but also helps to eliminate the possibility that some specific water structure, such as a linear polymer, is responsible for aligning the molecules with respect to the polypeptide.

Comparison of the spectra with those of water complexed with various bases in carbon tetrachloride is instructive. Water in pure carbon tetrachloride has two OH stretching bands ν_3 the antisymmetric mode at 3706 cm^{-1} and a weaker ν_1 symmetric band at 3615 cm^{-1}. When a 1:1 association of the type H-O-H..B forms the symmetry is lost, and bands occur at about 3690 cm^{-1} (free) and 3610 to 3550 cm^{-1} (bonded), the free OH being the stronger and narrower. With a 2:1 complex B...H-O-H...B, the symmetry is restored with bands at 3670-3550 cm^{-1} (antisymmetric) and 3580-3490 cm^{-1} (symmetric), of about equal intensity and breadth.[13] This suggests that the water in the polymers resembles the 2:1 complex. For poly Ala the only reasonable sites are peptide groups, where the lone-pair orbitals on the oxygen atoms are available to form bonding sites. Possibly a water molecule forms a bridge between two consecutive peptide oxygen atoms. The geometry of the helix does not produce a perfect fit, but since the two atoms are about 3Å apart weak hydrogen bonds might be formed giving rise to the two bands observed. The spectra of methanol and ethanol adsorbed on poly Ala

appear to show only one band in the CH-stretching region, unresolved from the Amide A band, again indicative of only one type of binding site. In contrast, alcohols produce two bands when adsorbed on the glutamate polymers, suggesting that there is an additional site, presumably on the side chain. This is consistent with the presence of a single band at 3559 cm^{-1} when ethyl acetate forms a 1:1 complex with methanol in carbon tetrachloride.[14] The component at ca. 3530 cm^{-1} might therefore be an OH-side chain interaction and that at 3450 cm^{-1} an OH-peptide group interaction as suggested for poly Ala. If water molecules adsorb on the same sites as alcohols on the glutamates, the band at 3570cm^{-1} may be a water-side chain interaction. The second hydrogen may form a bridge to a peptide group and models suggest there are several possibilities of this type. In addition water may bind to the peptide groups in the same way as in poly Ala.

While the precise assignment of the interactions of water with the peptide group and side chains requires further study, it is clear from the results that both types of site can be involved and from the frequencies, the hydrogen bond component of the binding is weak. Since the rest of the spectrum shows no detectable change, there seems little possibility that the normal peptide hydrogen bonds are opening. The fact that when using N-deuterated polymers, back exchange of deuterium is slow, supports this view. In seeking a more rigorous analysis of the frequencies it should however be noted that while in an isolated helix all

residues may be considered equivalent, in a crystalline lattice that is no longer true and several types of environment may occur for a water molecule binding to a chemical group, with corresponding energies and frequencies being involved. The orientation of the water cannot be related precisely to that of the helices, until it is clear whether the bonding enables us to consider the vibrations to be perturbed ν_1 and ν_3 modes with transition moments parallel and perpendicular to the symmetry axis, or whether the hydrogen atoms are sufficiently decoupled for them to move independently with moments along the bond directions. If the former possibility is correct, then assigning the dichroism to ν_3, it is possible to relate the H-H axis of the water to the orientation of the polymer. This is not very exact, since the orientation of the polymer.and resolution of the bands is poor, and a detailed discussion is not therefore warranted at this stage, but it appears that the H-H direction is within about 25° to the plane perpendicular to the molecular axis.[6] Since however all components of the bands appear to show perpendicular dichroism, it is reasonably certain that the planes of the water molecules (which contain the stretching modes, irrespective of the precise directions of the transition moments in the plane) lie in planes more perpendicular than parallel to the molecular axis.

There are surprisingly few observations of a similar nature on related systems, particularly in view of the importance of protein-water interactions. Bendit[15] has however observed weak perpendicular dichroism of water adsorbed on hydrated α-keratin. The character of the dichroism

and the fact that the peak of the absorption band is 30-40 cm^{-1} higher than liquid water are consistent with the results reported here, and his tentative conclusions for the positions of the water molecules in the crystalline regions are similar. The spectrum otherwise resembled that of liquid water.

Observation on the specimens prepared from mixed monolayers of 1:1 poly Ala, poly Glu(OMe) show dichroism in the water band as high as that of either polymer individually, but the band becomes smoother as in liquid water. Thus Bendit's observation that the shape of the water adsorption band resembled liquid water is probably not an indication of the state of the water, but rather a consequance of the complex structure of the keratin producing a range of somewhat differing sites for adsorption of water on to the peptide groups.

CONCLUSION

It will be seen that there is a close parallel between the type of interactions required to explain the surface potential results and the infrared spectra. The surface potential of α-helices with hydrocarbon side chains is not sensitive to the size of the side chain and must arise mainly from an interaction of water with the helix backbone. The positive sign of the potential requires that on average the water dipoles have a larger positive component directed upwards than on a clean water surface. This is consistent with water molecules reorientating with hydrogen atoms directed towards the lone pair orbitals of the peptide oxygen atoms.

The infrared results on poly Ala suggest a similar type of interaction. When ester groups are present in the side chains, the surface potential no longer shows a regular pattern of behaviour and a higher potential (at the same area/residue) is observed than in poly Ala. Specific interactions of water with the side chains are required to account for both this and the increased complexity of the infrared spectra.

In this simplified approach to the origin of the surface potential, we neglect any contribution from water adsorbed at the polymer-vapour interface which the infrared results suggest is strictly incorrect, but which can perhaps be regarded as small and approximately cancelling a part of the contribution at the polymer-liquid interface. It must also be recognised that the amount of water adsorbed in the infrared studies is small, while water molecules involved in polymer-water interactions, giving rise to the surface potentials, are probably equally involved in water-water interactions, Furthermore both the infrared and surface potential techniques present a time averaged picture of the interactions. While water adsorbed on thick films is relatively static and probably resembles water of crystallisation, at the polymer-liquid interface undoubtedly the water is more mobile and the potential is an average in space and time of the situation at individual sites. In seeking to relate the two different types of observation these considerations are important. However, in view of our present limited understanding of the surface potential, general correlations between it and other types of observations are valuable. That a correlation appears possible probably arises from the short range nature of the interactions between the polymer and liquid water.

There is now a considerable amount of evidence consistent with the view that many high molecular weight polypeptides are stable in the α-helical conformation at the air-water interface, both from direct observations on monolayers and by a diversity of indirect methods.[2-5, 17,18] Early workers favoured extended chain conformations and a major factor in this assignment was the surface potential which they attributed to the orientation of the peptide groups in the interface[1]. The present work is good evidence that the potential can arise entirely from water-polymer interactions and a fairly self-consistent pattern emerges. Our understanding of the origin of the potentials is of value not only in interpreting polymer-water interactions, but as a tool enabling us to study the monolayer in the unperturbed state to follow conformational changes and interfacial reaction.

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Captions for Figures

Figure 1. Measurements of surface pressure and potential as a function of area for polymers spread on 0.01 M KCl, 20°C. In each of the four sets of observations the side chain increases by $-CH_2-$ with successive letters of the alphabet. (a) Poly-L-alanine, (b) Poly-L-α-amino-n-butyric acid, (c) Poly-L-norvaline, (d) Poly-L-norleucine, (e) Poly- δ - benzyloxycarbonyl-L-ornithine, (f) Poly- ε - benzyloxycarbonyl-L-lysine, (g) poly-β-benzyl-L-aspartate (h) Poly-γ-benzyl-D-glutamate, (i) Poly-γ-methyl-L-glutamate, (j) Poly-γ-ethyl-L-glutamate.

Figure 2. Polarized spectra of water and alcohols adsorbed from the vapour on orientated films of (left) Poly-L-alanine. (right) Poly-γ-ethyl-L-glutamate, 30°C. The orientation of the electric vector is shown with respect to the axes of the helices. The OH-stretching absorption ($3400-3600^{-1}$) is always stronger with the electric vector perpendicular to the helical axis. The zero of the scale in this region has been displaced as shown by the horizontal lines, for different treatments.
In the case of poly-L-alanine the spectra of polymer exposed to methanol vapour are marked V. At 3300 cm^{-1} VI by chance coincides with the peak height of the untreated film with the electric vector parallel.

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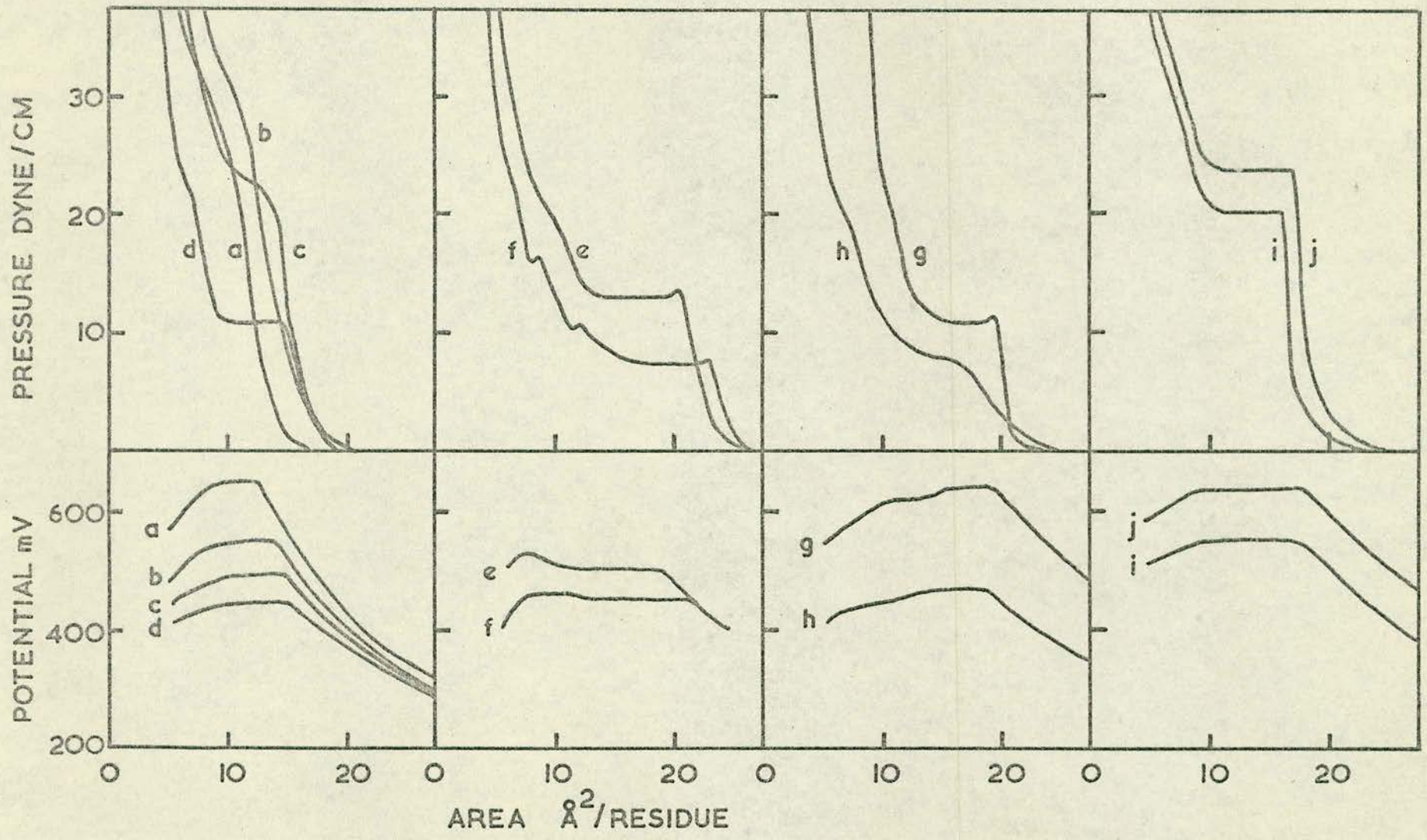
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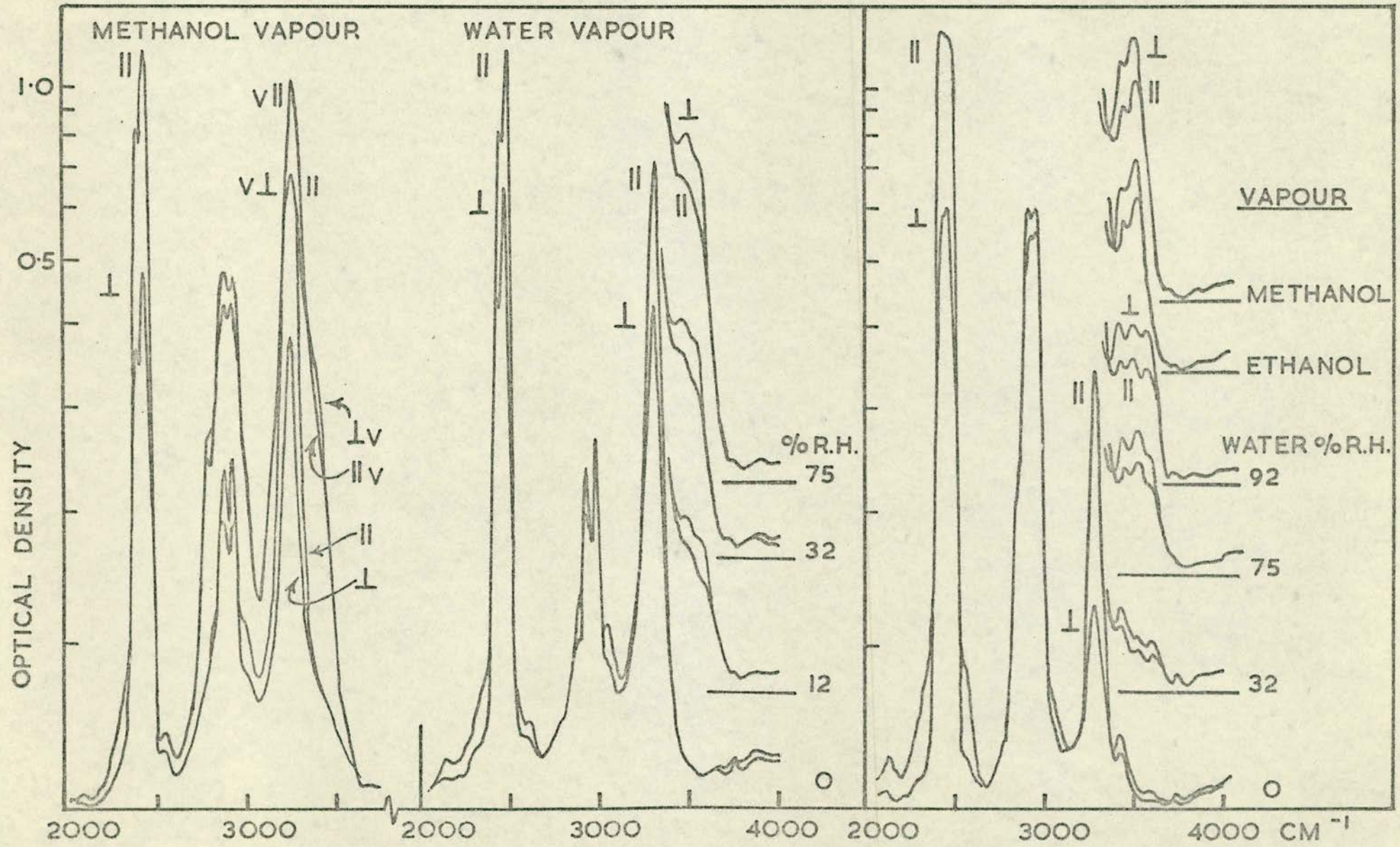
Figure 1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IIν
%R.H.
75
32
12
0
METHANOL
ETHANOL
WATER %R.H.
92
2000
3000
4000
CM⁻¹

Figure 2